STUDIES ON THE MOLECULAR STRUCTURE OF STARCH - TYPE POLYSACCHARIDES

- by -

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TO MY PARENTS.

A C K N O W L E D G E M E N T S.

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SECTION 1.

GENERAL INTRODUCTION.

Starch is the major reserve polysaccharide of the higher land plants although it is also known to occur in protozoa (1), algae (2), and in bacteria (3). It forms the principle food source of man and exists in Nature as discrete, weakly birefringent granules, often nearly spherical in shape, which readily stain blue-purple with iodine. These granules appear to consist of at least two chemically distinct polysaccharides although the possibility of intermediate types between these two cannot be ruled out (4). Both these polysaccharides consist of glucose units, but differ from each other in that one appears to be linear (or nearly so) while the other is highly branched. Amylose, the linear component of starch, which can be separated from amylopectin, the branched component of starch, as an insoluble complex by the addition of an alcohol of low solubility, consists of glucose residues which are linked to each other through positions 1 and 4 to form a polymer of high molecular weight (ca. 1,000 to ca. 1,000,000). The α-configuration of these glucosidic bonds, indicated by the high positive optical rotation of amylose solutions, has been confirmed by partial hydrolysis giving maltose (4-O-α-D-glucopyranosyl-D-glucose), and higher oligosaccharides which have the α-configuration. The structure /

structure of amylose has been confirmed by methylation and periodate oxidation studies and by biochemical techniques involving the controlled enzymic hydrolysis of the polysaccharide using purified enzyme preparations. It is these latter techniques which, while confirming the general evidence of the other methods, have provided evidence as to the fine structure of the amylose molecule.

β-Amylase, an enzyme found in the higher plants, effects a step-wise degradation of the amylose molecule hydrolysing alternate α-1,4-linkages with the liberation of maltose. Early work with this enzyme gave β-amylolysis limits of ca. 100% (i.e. 100% conversion to maltose) indicating that amylose was a linear molecule. Peat and co-workers (5) have shown however that highly purified preparationsof this enzyme give lower β-amylolysis limits (ca. 70%) indicating that there are a small number of barriers to the action of this enzyme. Recent work has been concerned with the nature of these barriers and with the nature of the impurity (Z-enzyme) in the less highly purified preparations of β-amylase which is capable of either hydrolysing or by-passing these barriers (6). It now seems that Z-enzyme, if not absolutely identical with α-amylase, acts in exactly the same manner to that enzyme in that it catalyses a limited random hydrolysis of α-1,4-linked glucosans exposing in the case of amylose additional polysaccharide material which is suitable for attack by β-amylase. /

β-amylase.

Research into the nature of the barriers in amylose to β-amylolysis, or anomalous linkages as they are generally named, has until recently provided little evidence of a positive nature. Suggestions by Gilbert and his co-workers (7) that these anomalies were introduced by accidental atmospheric oxidation during the isolation of amylose have been shown by Manners and his co-workers (8) to be incorrect in that the anomalies are a structural feature of the amylose molecule, and not artefacts arising from preparative procedures. Both ester phosphate groups (9) and single glucose side chains (10) have been considered as possible sources of the anomalous linkages. Manners and Kjölberg (11), however, have recently shown that the barriers to β-amylase are a small number of α-1, 6-linked branch points in the molecule. This result is in accordance with known facts concerning the bio-synthesis of starch and does not infer unknown properties on the known enzyme systems or postulates the existence of new enzymes. Amylopectin differs from amylose in that, although the main chain forming linkage is also α-1,4, there occur a number of α-1,6-linkages which act as branch points. The general structural features mentioned have been obtained by various chemical techniques - periodate oxidation (12), partial acid hydrolysis (13) and methylation (14). The branched structure of amylopectin has been the subject of considerable research /

research in attempts to discover the arrangement of the chains in the molecule. The tree-type structure first put forward by Meyer (15) is now generally accepted principally on the basis of biochemical evidence (16) although the original concept arose from methylation studies.

The branched structure of amylopectin will obviously give rise to a different β-limit from the almost linear amylose molecule since β-amylase is incapable of hydrolysing α-1,6-linkages. This incomplete β-amylolysis (ca. 50-60% conversion into maltose) gives as an end product a β-limit dextrin of high molecular weight which contains short chain stubs of 2-3 glucose units. Periodate oxidation of the amylopectin molecule and measurement of the formic acid produced gives a measure of the average chain length (C.L.) of the molecule (ca. 20). This knowledge in conjunction with the β-amylolysis limit gives a measure of the lengths of the exterior and interior chains of the molecule.

Glycogen, sometimes referred to as animal starch, in that it constitutes the reserve polysaccharide of the animal kingdom, has obvious similarities to amylopectin. It is an α-1,4-linked glucose polymer with α-1,6-linked branch points. It differs from amylopectin in that it has a shorter chain length (C.L. 10-15) and a lower β-amylolysis limit (ca. 40-50%) indicating a more compact molecule and giving rise to differences between the two groups of polymers in /

in their physical properties.

Scope of the Present Work.

The present work is concerned mainly with the re-evaluation of the evidence for certain minor structural features in amylopectin including the probable occurrence of less than 0.5% α-1,3-linkages in the molecule, and the possibility that fructose is a minor constituent. Further studies are included on the iodine staining of starch type polysaccharides, and the possible relationship to the structure of these polymers, and, on the structure of the iodophilic polysaccharide of the protozoa Polytoma uvella.

SECTION 2.

GENERAL METHODS.

1. Paper chromatography:- For the separation of di- and higher saccharides both ascending and descending chromatography were used.

Solvents (1) Ethyl acetate - pyridine - water (10: 4: 3 v/v)

(2) Butanol - pyridine - water (6: 4: 3 v/v).

(3) Methyl ethyl ketone - acetic acid - water (9: 1: 1 v/v), saturated with boric acid.

(4) Butanol - ethanol - water (40: 11: 19 v/v).

Spray Reagents

(1) Alkaline silver nitrate: The paper, either air dried or dried at 80°C., was sprayed with a solution of silver nitrate in aqueous acetone. The solution was prepared by the addition of 1ml. saturated aqueous silver nitrate to 200ml. acetone and dissolving the resultant precipitate by the dropwise addition of water. The paper was then dried and sprayed with a sodium hydroxide solution prepared by adding 10ml. 5N.NaOH to 40ml. ethanol. The very light background colour produced was removed by dipping the paper in a saturated solution of sodium thiosulphate and then washing thoroughly with water.(17)

(2) Aniline oxalate: The dried paper was sprayed with a saturated solution of aniline oxalate in methylated spirits and the paper heated at 120°C. for 10 minutes. (18)

(3) /

(3) o - Amino diphenyl: The dried paper was lightly sprayed with the reagent and heated 1 - 2 minutes at 120°C. The reagent was made up as follows:- 3.0g. o - amino diphenyl, recrystallised from aqueous ethanol, was dissolved in 100 ml. glacial acetic acid to which was added 1.3 ml. of 85% phosphoric acid (19). This spray appears to be much more sensitive than aniline oxalate but its use was discontinued on discovering that it was a highly active carcinogen.

(4) Potassium periodateocuprate: The paper was first sprayed with rosaniline solution, air dried for 5 minutes, then sprayed with cupriperiodate solution. (20). The reagents were made up as follows:-

(a) Rosaniline solution:- 0.3g. rosaniline base in 100ml. acetic acid diluted to 1000ml. with acetone.

(b) Periodateocuprate:- 12.5g. copper sulphate pentahydrate dissolved in boiling water. 23g. potassium periodate was added followed by 56g. potassium hydroxide in concentrated solution. 20g. of potassium persulphate were then added in small portions at one minute intervals and the solution then boiled for 20 minutes. After cooling, the solution was decanted, 500ml. 2N potassium hydroxide added and then water to a final volume of 1 litre.

The cupriperiodate reagent on its own appears to be rapid and sensitive although the white spots it produces /

produces on a brown background are impermanent. The use of the rosaniline spray, in addition, reduces the apparent sensitivity while giving very pale pink permanent spots.

(5) Lead tetra acetate-rosaniline: The paper was dipped in 1% solution of lead tetra acetate in chloroform, air dried then sprayed with the rosaniline solution as in Reagent 4 and heated in a current of warm air (21). This reagent is more sensitive than Reagent 4.

(6) Periodate - permanganate: The paper was sprayed with the reagent freshly prepared before use by mixing 4 parts 1% aqueous sodium metaperiodate and 1 part 0.5% potassium permanganate in 2% aqueous sodium carbonate. The sprayed paper was suspended in a current of warm air for 10 minutes and then allowed to develop at room temperature for a further 30 minutes before washing with water giving brown spots on a white background (22).

(7) Orcinol: Specific for ketoses (23). Sprayed with freshly prepared reagent (0.5 g. orcinol, 15 g. trichloroacetic acid, 100ml. water saturated butanol) and then heated at 105°C for 15-20 minutes.

2. Charcoal column chromatography:- Quantitative, large scale separations of oligosaccharides were carried out on charcoal-Celite columns (24) using Ultrasorb 120-240 charcoal (25) and Celite "545". Equal weights of charcoal and Celite were mixed dry and then made up to a thick slurry with water which was slowly poured into a glass column onto

a /

a pad of Celite ($\frac{1}{8}$"-1" thick) supported on glass wool. After the addition of the slurry the column was washed thoroughly with distilled water. The oligosaccharides were eluted by allowing suitable solutions of aqueous ethanol to pass through the column.

3. Iodine staining:

(a) Aqueous solutions:- a solution containing 2.5mg. glycogen or 1.25mg. amylopectin was pipetted into a 25ml. standard flask together with 2.5ml. of an iodine solution containing 0.2% iodine and 2% potassium iodide and the volume adjusted to 25ml. by the addition of distilled water. The absorption of the solution was compared with an iodine water blank in a Unicam S.P500 or S.P600 spectro-photometer between 430-540mμ for glycogens and 470-580mμ for amylopectins.

(b) Salt solutions:- a solution containing 2.5mg. glycogen or 1.25mg. amylopectin was pipetted into a 50ml. graduated flask along with 5.0ml. of the above iodine solution and 25ml. of a saturated solution of the salt and the volume adjusted to 50ml. with distilled water. The absorption was compared with an iodine salt blank as above.

4. Estimation of reducing sugars:- This was carried out using the Somogyi (1952) reagent (26) or the Shaffer Hartman reagent (27). 5Ml. of the reagent was heated at 100°C on a water bath for 20 minutes with a suitable quantity /

quantity of the sugar (0 - 3mg. monosaccharide, 0 - 6mg. disaccharide). The solution was cooled in cold water, 3ml. of 2N. sulphuric acid added to it, and the liberated iodine titrated with 0.01 N. sodium thiosulphate. The difference between the observed titre and a blank titre obtained from 5 ml. of the reagent alone is a measure of the quantity of reducing sugar present. The exact quantity of sugar present can be read from a calibration graph, of titre difference against mg. sugar, prepared from standard sugar solutions whose concentration was determined polarimetrically.

5. β-amyloysis :- Two commercial preparations of amylase were used in this work.

(1) Purchased from Wallerstein laboratories (New York). Enzyme activity was 100 units/mg. as determined by the method of Hobson Whelan and Peat (28) and the enzyme, though free from maltase, contained a trace of Z-enzyme.

(2) Purchased from the Worthington Biochemical Corporation (Freehold, New Jersey). The suspension of crystals supplied was diluted with 0.06 mM glutathione (29) to give a stock solution with an activity of 200 units/ml. The enzyme was free from maltase and Z-enzyme. Enzyme digests using this enzyme required the addition of serum albumin and glutathione to stabilise the enzyme. A "digest base" containing these substances and also buffer for inclusion in the digests was made up to the following final

composition /

composition :- buffer 0.2M, glutathione 0.5mM, and serum albumin 0.5% (W/v). 1ml. of this solution was included in each 10 ml. of the digest.

6. Acid hydrolyses:-

(a) Total acid hydrolyses:- The exact concentration of polysaccharides in solutions was determined by total acid hydrolyses of approximately 2mg. of the polysaccharide using 2N. sulphuric acid for 3 hours at 100°C. The solution was neutralised with 10N. sodium hydroxide and then 0.2N. sulphuric acid was added until the solution was just acid to phenolphthalein, and the monosaccharides obtained estimated by the Somogyi (1952) reagent.

For qualitative examination of the products of an acid hydrolyses the solution was neutralised with solid barium carbonate, the resultant precipitate centrifuged off and the supernatant liquid evaporated to dryness under vacuum at 35°C. The residue from the evaporation was extracted twice with 70% aqueous ethanol and then concentrated before examination by paper chromatography.

(b) Partial acid hydrolysis:- The formation of oligosaccharides from polysaccharides was effected by heating the polysaccharide with 0.33 N. sulphuric acid for ½ - 1 hour. The resultant solution was neutralised with barium carbonate and extracted with aqueous ethanol as above.

7. Electrophoresis:- The separation of mono- and disaccharides was carried out at 750 volts and a final

current /

current density of circa. 10 milliamps in borate buffer (30) and in germanate buffer (31) at pH 10.7 on an apparatus similar to that of Foster (32). Whatman No. 1 paper was used. The papers were dried at 80°C., and the sugars located by spraying the paper with a saturated aqueous solution of aniline oxalate to which was added an equal volume of glacial acetic acid, and then heating at 120°C. for 10 minutes.

8. Periodate Oxidation:-

(a) Determination of formic acid release.

Two methods were used for the estimation of formic acid, both of which give the same result, although perhaps the second is the easier of the two to use and gives the sharper end point:

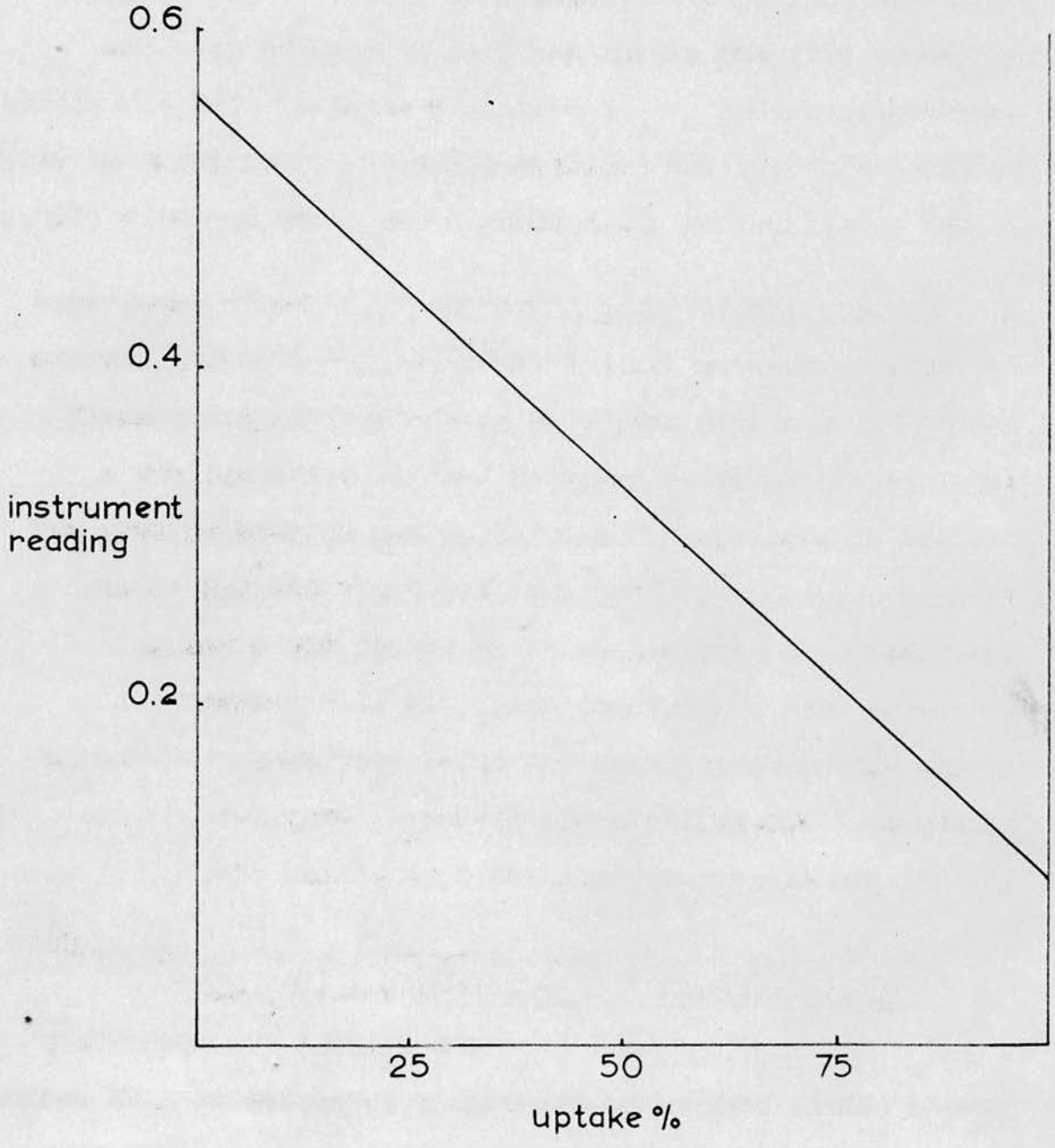
(i) A suitable aliquot of the reaction mixture was reacted with 2ml. ethylene glycol in the dark for 20 minutes and then titrated with 0.01N. carbonate-free sodium hydroxide under an atmosphere of nitrogen to pH 5.8 using a Pye Universal pH meter.

(ii) A suitable aliquot of the reaction mixture was reacted in the dark with 2 ml. ethylene glycol for 20 minutes. To this solution was added 2ml. neutral, freshly dissolved, 10% (v/v) potassium iodide and the solution titrated with 0.005N. sodium thiosulphate using a 1% solution of starch or starch glycollate as indicator.

(b) Periodate uptake was measured by the method of Aspinall and Ferrier (33) in which an aliquot of the reaction mixture was diluted to approximately 6×10^{-5}M. and its absorbance at 222.5 mμ measured against a water blank in a Unican SP 500 spectrophotometer. The periodate uptake /

Figure 1.

Periodate uptake calibration graph



uptake was calibrated using a periodate solution of the exact concentration of the reaction mixture and removing two aliquots from this solution. One was diluted in exactly the same way as the reaction mixture while in the other the periodate was reduced to iodate by the addition of excess ethylene glycol and then diluted to give the same concentration as the periodate sample. The absorbance of both solutions was measured against a water blank at 222.5 mμ and a calibration graph prepared as shown opposite (Fig.1).

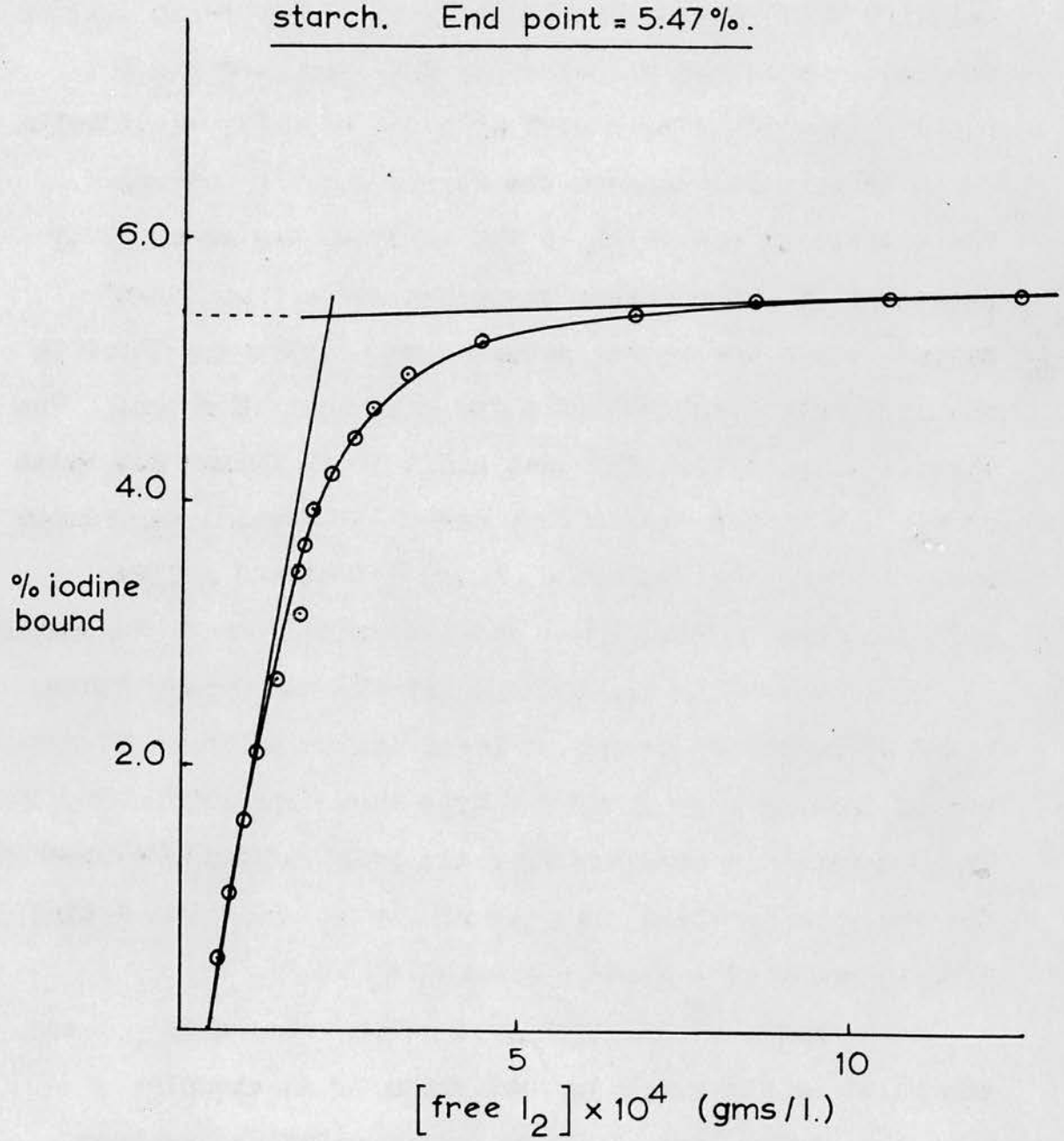
9. Preparation of Sugar Acetates:- 10 Parts sugar were heated with 5 Parts freshly fused anhydrous sodium acetate and 70 Parts acetic anhydride at the boiling point until solution was effected and then heating continued for a further 10 minutes. The solution was allowed to cool and then poured with stirring into 500 Parts ice and water. The mixture was allowed to stand at 2°C. for 4 hours, extracted with chloroform, which was then shaken with sodium carbonate solution and dried over anhydrous sodium sulphate. The solution was filtered, evaporated to dryness and the residue re-crystallised from ethanol (34).

10. Estimation of the Amylose Content of a Starch by Potentionmetric Iodine Titration. (35, 36).

Starch (ca. 40mg.) was weighed out accurately into a 250ml. beaker and thoroughly dispersed in 0.5N sodium hydroxide (10ml.). After dispersion, which usually took 3 - 5 hours, 0.5N hydrochloric acid was added until the solution /

Figure 2.

Potentiometric iodine titration of Chlorella vulgaris starch. End point = 5.47%.



solution was just acid to methyl orange and then 0.5M potassium iodide solution (10ml.) and distilled water (70ml.) added to give a final volume of solution of 100ml. with a potassium iodide concentration of 0.05M. The solution was titrated at room temperature with 0.00 1M. iodine solution which was 0.05M. with respect to potassium iodide. The iodine solution was added in 2ml. portions and 5 minutes allowed between each addition to allow equilibrium to be established between the starch and the iodine. The electrical potential of the solution was measured by placing a bright platinum electrode and a "saturated" calomel electrode in the solution and connecting these to the millivolt terminals of a Pye Universal pH meter. The electrical potential for each addition of iodine was noted at the end of the five minute period. A blank experiment using exactly the same acid, base, iodine and iodide solutions but no starch was carried out. The concentration of "free" iodine is plotted against the percentage iodine bound (measured as weight of bound iodine in gms./100 gms. starch) giving a graph of the type shown opposite. (Fig. 2). The end point is obtained from the point of intersection of the two straight line portions of the graph. The iodine binding power of amylose was taken as 19.5%.

Notes on calculation:- The calculation of any one point on the graph is best shown by an example.

After 10ml. 0.0010M. iodine solution has been added to a solution containing 40.0mg. of a starch the

e.m.f /

e.m.f. = 236 m.v. This e.m.f. corresponds with that produced by the addition of 2.5ml. 0.001N iodine in the blank experiment obtained by graphing m.v. readings against ml. iodine added.

"Total" iodine concentration = $\frac{10}{100+10}$ x 0.001M = [Total]

"Free" iodine concentration = $\frac{2.5}{100+2.5}$ x 0.001M = [Free]

"Bound" iodine concentration = [Total] - [Free] = [Bound]

Starch concentration = $\frac{40}{110}$ mg/ml.

% I_2 bound = $\frac{[\text{Bound}]}{40}$ x 110 x 100

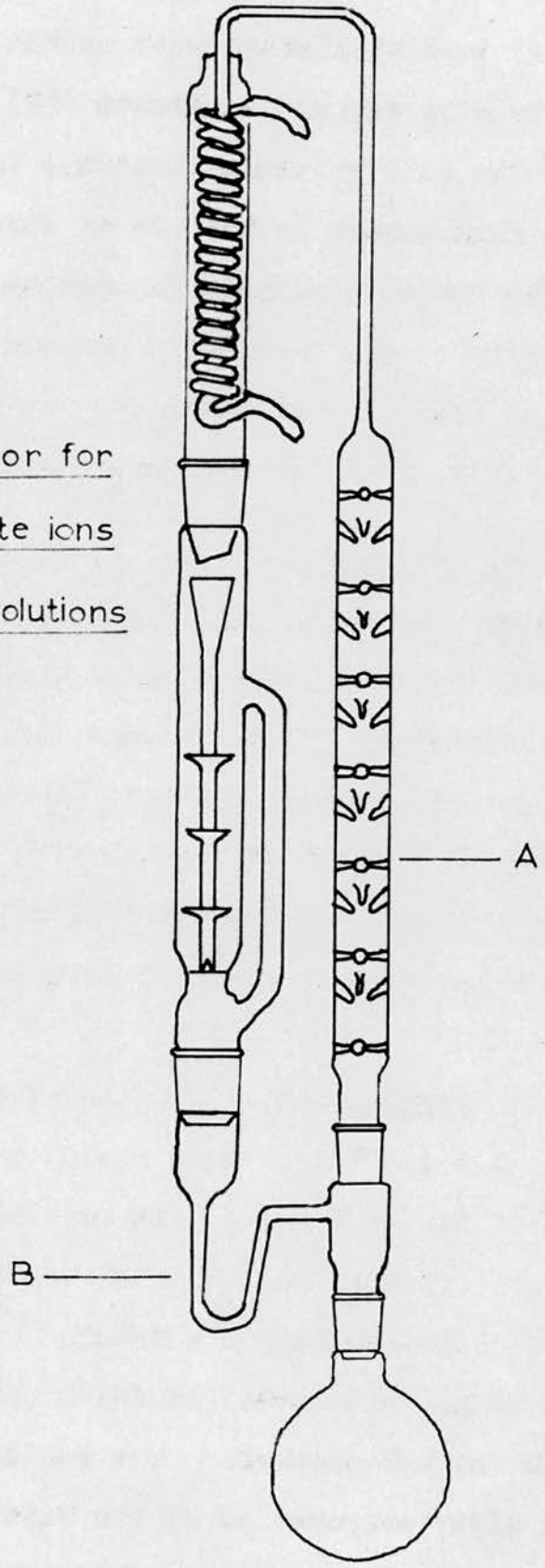
11. The Removal of borate ions from carbohydrate solutions

The use of the borate complexes of sugars as an aid to the separation of mixtures of oligosaccharides both by electrophonesis (30) and charcoal chromatography (37), is well established but fuller use of this technique has not been made due to the tedious methods of removing the borate ions from solution. The use of an anion exchange resin is not suitable since the resin cannot be used in the carbonate form (which does not harm sugars) since boric acid, having a lower dissociation constant than carbonic acid, is not absorbed. The use of an anion exchange resin in the hydroxyl form chemically alters reducing sugars (38). Di- and higher saccharides can be freed from ions by absorbing them on the laboratory grade of activated charcoal while monosaccharides can be similarly deionised by the use of more active grades of charcoal (25). The removal of

boric /

Figure 3.

Liquid-liquid extractor for the removal of borate ions from carbohydrate solutions



boric acid by co-distillation with methanol has unfortunately been shown to give anomalous results (39) and it would thus appear that the only suitable procedure for the removal of borate ions from sugars is the use of charcoal columns.

The use of a solvent extraction technique for the removal of borate ions from sugar phosphates has recently been reported (40). This technique has now been applied to the removal of this ion from mono- and di-saccharides.

Description of apparatus:- (Fig. 3) Isoamyl alcohol, approx. 250 ml., is boiled in a 500 ml. round bottomed flask and the vapour allowed to pass upwards through a vigreux fractionating column (A) and then down into a single spiral condenser. The cold liquid emerging from the condenser is allowed to pass through the aqueous phase in the form of small bubbles and then overflow through an S-bend (B) which acts as a vapour lock and back into the boiling flask.

Extraction of borate ions:- 40 Ml. saturated aqueous acid (solubility 5.2 g/100 ml.) were placed in the apparatus and extracted for 10 hours. The resulting liquid was evaporated to dryness and the total remaining boric acid was found to dissolve in 5 ml. water. This solution was diluted to 50 ml. and extracted for a further 10 hours using fresh isoamyl alcohol. The residue from this extraction after evaporation of the water dissolved in less than 0.5 ml. distilled water. This extraction was carried out /

out using a plain glass tube insulated with asbestos string in place of the fractionating column (B). Further experience showed that the borate ion is removed with the formation of tri-isoamyl borate and that the efficiency of the extraction was reduced as the ester accumulated in the boiling flask. It is thus presumed that the borate ion is removed as its tri-ester and that the presence of appreciable quantities of this ester in the vapour, which eventually forms the organic extraction phase, rapidly establishes a condition of equilibrium between the borate in the aqueous and the organic phases, with a concommitant reduction in the efficiency of the apparatus. The fractionating column was introduced into the apparatus therefore to reduce the amount of tri-isoamyl borate cycling through the apparatus to an absolute minimum.

Removal of sugars by solvent extraction:- Sugar solutions were acidified to pH4 with 0.1N sulphuric acid.

(1) Maltose:- approximately 50 mg. maltose was dissolved in 50 ml. distilled water in a graduated flask. 4 Ml. of this solution was removed to estimate the exact maltose content of the solution. The remaining solution was washed from the flask into the apparatus with distilled water and extracted for 72 hours. The aqueous layer was removed, reduced in volume, transferred to a 50 ml. standard flask and made up to the mark. A 4 ml. aliquot was again removed and the maltose content estimated by the Shaffer /

Shaffer - Hartmann reagent.

Maltose content before treatment	0.997 mg./ml.
Maltose content after treatment	0.883 mg./ml.
∴ percentage loss in 72 hours	3.7%

Paper chromatography of the maltose solution after extraction showed that only maltose was present in the solution.

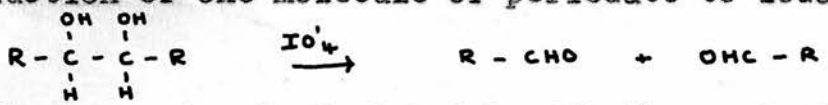
SECTION 3.

PART 1.

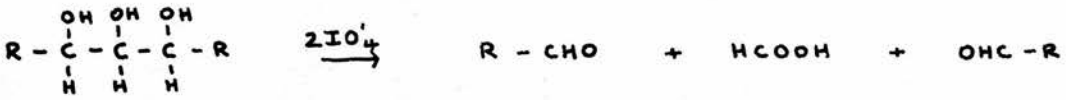
Evidence for 1, 3-linkages in starch from periodate oxidation studies.

INTRODUCTION

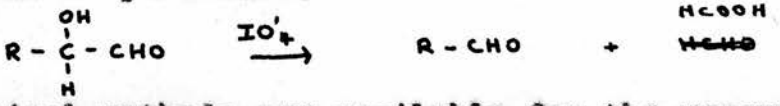
Oxidation using the periodate ion, first introduced by Malaprade (41), has become one of the major analytical tools of the carbohydrate chemist. The reaction involves the rupture of the carbon-carbon bond of 1, 2-glycols with the production of two aldehydic groupings and the reduction of one molecule of periodate to iodate.



or in the case of a 1, 2, 3-triol with the production of one molecule of formic acid, two aldehydic groups and the reduction of two molecules of periodate.



The oxidation of an α-hydroxy aldehyde produces one aldehyde group, one molecule of ~~formaldehyde~~ formic acid and reduces one molecule of periodate.



Analytical methods are available for the measurement of periodate uptake, formic acid and formaldehyde release, as well as for aldehyde content, and, provided that the conditions for the reaction are suitably chosen, considerable information can become available concerning the structure /

structure of the mono-, oligo-, or poly-saccharide under study.

Two types of periodate oxidation reaction may occur. The first, which may be termed "selective", generally proceeds quickly and quantitatively giving after a period of time constant values for periodate uptake and for the formation of oxidation products. The second type shows no marked levelling off at a maximum value and is generally referred to as "overoxidation". In all but a few cases it is the selective oxidation which is desired and reaction conditions are chosen such that the amount of over-oxidation which occurs is kept to a minimum. Temperature, hydrogen ion concentration, absence of light concentration of reactants and products, as well as the use of periodic acid or one of its salts, all seem to exert an influence on the course of the reaction (42).

The application of analytical techniques to the periodate oxidation of polysaccharides has produced a considerable volume of information as to their structure. This is particularly the case with regard to glucose polymers containing 1, 4-linkages, with or without the presence of other linkages as part of the main chain, or with 1, 6-linked branch points. Thus Hirst et al. (43) showed, by comparison with methylation analysis, that measurement of the formic acid liberated by the periodate oxidation of amylopectin indicated that few, if any, of the 1, 6-linkages

were /

were not involved as branch points in the molecule.

The measurement of the formic acid released by periodate oxidation of amylopectin provides information as to the average length (C.L.) of the 1, 4-linked chains of glucose molecules since the formic acid produced by the reducing end group is insignificant compared with that produced by the non-reducing end groups of the molecule. This information in conjunction with β-amylolysis of the polysaccharide gives the average length of the exterior and interior chains of the molecule.

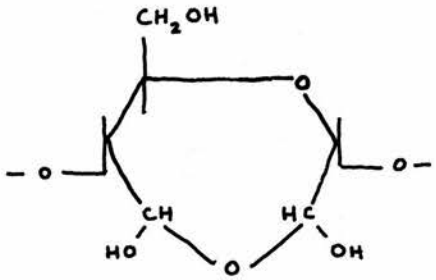
Periodate oxidation of a glucose polymer containing only 1, 4- and 1, 6-linkages should destroy all the glucose residues. However, the presence of 1, 2- or 1, 3 -linkages in the polymer will result in glucose units which are not attacked by periodate, and de-polymerisation of the oxidised material should produce free glucose which can be estimated and characterised. The acid hydrolysis of the residual material after the determination of the average chain lengths of starches, amylopectins and glycogens by several workers has resulted in the observation of small amounts of glucose (0-2% of the original polysaccharide). The possibility therefore arises that this may be due to the presence of a small percentage of 1, 2- or 1, 3- linked glucose units. The interpretation of these results as showing conclusively the presence of these minor linkages is /

is difficult since there are several factors which can invalidate these results and any, or all, of these may be operative in the determination of the residual glucose.

Incomplete oxidation of the polymer will give residual glucose and it is perhaps worthwhile noting that the occurrence of overoxidation in a periodate oxidation may not give an exact guide to the completeness of the oxidation in the entire molecule. It is quite possible for a considerable number of glucose units in a polymer to be unattacked yet over-oxidation takes place.

It is known that in 1, 2 diols with the hydroxyl groups locked in the trans position periodate attack cannot occur (44), and it is possible in a high polymer of the type being considered that glucose units are held in such a conformation that periodate attack is hindered or prevented (45).

Investigation of the dialdehydes produced by oxidation of amylopectin with periodate (46) has shown that these compounds probably exist in the hemialdal form i.e.



Thus two of the aldehyde groups formed react together /

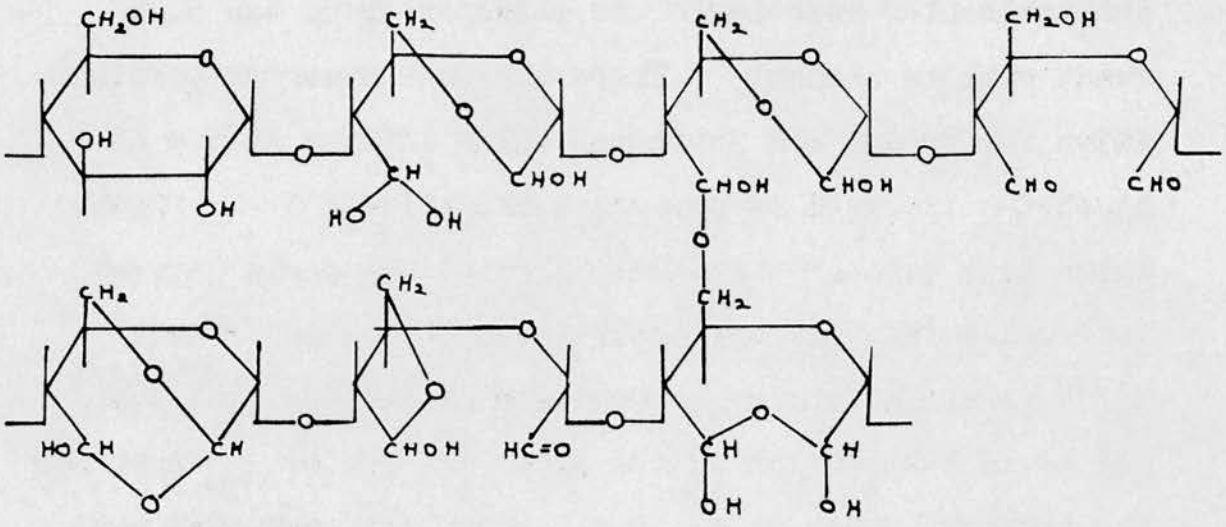


Figure 4.a. Some possible polyaldehyde structures

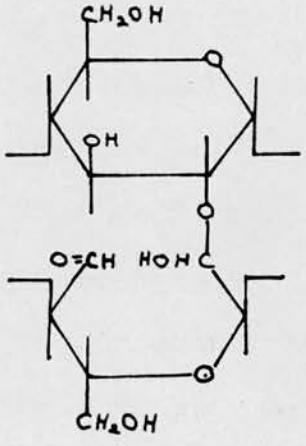


Figure 4b Hemiacetal formation involving unattacked glucose units

together and this need not necessarily be confined to intramolecular reaction. An aldehyde group can also react with an alcohol. There are thus numerous possibilities for inter- and intra-molecular linking in the dialdehydes produced on periodate oxidation (47). Figure 4a shows only some of the possibilities, there are several intermolecular linkings possible which are not shown. It is feasible, during a periodate oxidation, that linking may occur between one of the aldehydic groups produced and the hydroxyl group on C_2 or C_3 of an anhydroglucose unit in the polymer which would render it resistant to periodate attack (Figure 4).

Glycollic aldehyde, one of the hydrolysis products of reduced starch, glycogen and amylopectin polyaldehydes, is known to polymerise to hexoses under mildly alkaline conditions (48), and its behaviour under conditions used for acid hydrolysis has not yet apparently been described. Glyoxal, a product of the hydrolysis of the polyaldehydes, presumably undergoes similar polymerisation in alkaline conditions and the products from both these compounds will have a similar mobility to glucose on paper chromatography. Should any, or all, the glyoxal or glycollic aldehyde persist after acid hydrolysis of a polyaldehyde, or polyalcohol, it is quite possible that carbohydrate material will be produced during the neutralisation process. Thus estimation of residual glucose after a periodate oxidation must /

TABLE 1.

Residual Glucose in Starches, Amylopectins and Glycogens.

Polysaccharide	Oxidation Conditions	Method of Estimation	% Glucose	Reference.
Barley Starch	1	A	1.8	(52)
Rubberseed Starch	1	A	1.2	(53)
Potato Starch	1	A	1.1	(43)
Acorn Starch	1	A	1.8	(43)
Sago Starch	1	A	1.2	(43)
Barley Amylopectin	1	A	0.6	(52)
Rubberseed Amylopectin	1	A	0.75	(53)
Maize Amylopectin	2	B	0.1	(51)
Maize Amylopectin β-dextrin	2	B	0.2	(51)
Commercial Glycogen	2	B	0.25	(51)
Cat liver Glycogen	3	C	0.00	(50)
Mussel Glycogen	3	C	0.00	(50)
Snail Glycogen	3	C	0.02	(50)
Tetrahymena Glycogen	3	C	0.00	(50)
Waxy Maize Starch	4	D	0.2-0.5	(45)

1. Sodium periodate + potassium chloride (57).
2. Sodium periodate at 5°C.
3. Potassium periodate (satd.).
4. See Text.

A. Somogyi reagent.
B. Aniline hydrogen phthalate.
C. Glucose oxidase.
D. Phenol - sulphuric acid.

must be carried out using a reagent specific for glucose, or, if a selective reagent is used for estimation, the carbohydrate material present must be shown to contain only glucose.

If there are a small number of linkages other than 1, 4 or 1, 6 in amylopectin there are indications that these linkages are 1, 3 rather than 1, 2 by the isolation of nigerose (3-O-α-D-glucopyranosyl-D-glucopyranose) from the partial acid hydrolysis of amylopectin and glycogen (See section 3. 2.). The latter would indicate an α-configuration for the linkages, a fact not revealed by the periodate oxidation studies.

Analysis of starch, amylopectin and glycogen polyaldehydes for residual glycose have been reported and a summary of the results obtained by several groups of workers is shown in Table 1. These results may be conveniently divided into three groups; whole starches, amylopectins and glycogens. The results of Hamilton and Smith (45) will be discussed separately.

The glucose remaining after the oxidation of unfractionated starches has been reported in amounts varying from 1.1 to 1.8% of the original material. It is probably safe to assume that these figures are attributable, to some extent at least, to the underoxidation of the polysaccharide since no attempt was made to exhaustively oxidise the polysaccharides. The values quoted are merely the /

the result of a residual glucose determination after oxidation had proceeded for a period of time which was suitable for the determination of the average chain length. Indeed, Anderson, Greenwood and Hirst (49) report that after oxidation in excess of this time they could only observe a glucose spot on a chromatogram of an acid hydrolysate of the oxidation product with the aid of ultraviolet light.

The residual glucose remaining after oxidation of amylopectins is less than that obtained from the whole starches in that values from 0.1 to 0.8% glucose have been reported although the granular nature of the starches may explain the difference in the values. Again little attempt has been made to exhaustively oxidise the polysaccharides, and, underoxidation and the method of estimation, which is not specific for glucose, may give rise to much, if not all, of the percentages quoted.

Bell and Manners (50) have carried out the prolonged oxidation of several samples of glycogen using potassium periodate for 40-50 days and have estimated the residual glucose by the use of D-glucose oxidase, a method which is virtually specific for glucose. Their results, which would appear to establish beyond reasonable doubt that glycogens are unlikely to contain 1, 2- or 1,3-linkages, are in contrast to that obtained by Gibbons and Boissonnas (51) for a commercial sample of glycogen.

It is rather unfortunate that those groups of workers who have taken the trouble to investigate the residual material after a periodate oxidation of starch or amylopectin did not prolong their oxidation times or attempt to use a specific reagent for glucose as did Bell and Manners though it must be said that none of these groups has claimed to have established the presence of 1,2- or 1, 3- linkages. This however is not the case with the investigation carried out on a sample to waxy maize starch by Hamilton and Smith (45) under conditions "in which every effort was made to eliminate the possibility of incomplete oxidation" and they claim that their results show that periodate resistant glucose units are present in the starch. In view of the claims of these workers it is necessary to consider their paper in some detail.

Oxidation of the starch was carried out using sodium periodate at 2-4°C and 26°C. for differing periods of time. The periodate uptake was measured by the sodium arsenite technique. After the destruction of excess periodate with ethylene glycol the starch polyaldehyde was reduced with sodium borohydride before hydrolysis with hydrochloric acid, deionisation, and estimation of the residual glucose, after separation of the components of the hydrolysate, using the phenol sulphuric acid technique. The results obtained are shown on the following page.

Time /

Time (days)	Temp. (°C.)	Starch (g.)	Reaction volume (ml.)	IO'_4 conc^n (M)	IO'_4 uptake (M/ahg)	Glucose %
5.5	2-4	5	250	0.2	1.04	0.22
48	2-4	20	1000	0.4	1.1	0.4
150	2-4	10	500	0.4	1.29	....
9	26	20	1000	0.4	1.5	0.52, 0.44

It is notable that the highest periodate uptake is accompanied by the highest percentage of residual glucose. This parallel for the low temperature (2-4°C.) oxidation is unexpected and it seems inconsistent with the ideas put forward, that, on oxidation of the same starch sample, it should have a higher residual glucose content after 48 days than after only 5.5 days. This is particularly so since one value is twice the other. There also seems to be little point in quoting the 150 day oxidation in this paper if no residual glucose determination was carried out. The high periodate uptake during the oxidation at 26°C. serves to illustrate the point made earlier that over-oxidation is no proof of complete "selective" oxidation.

Some of the polyaldehyde from the 150 day oxidation was reduced with sodium borohydride and then re-oxidised for 15 days with 0.4 molar sodium periodate at 2-4°C. when it showed a formic acid release of 0.9 mole formic acid per anhydroglucose unit and the odour of formaldehyde could be detected. After the removal of excess periodate, reduction with sodium borohydride and hydrolysis, glucose was identified in the hydrolysate by its mobility /

mobility on a chromatogram. Although the authors had isolated 6 g. of the polyaldehyde, and had used only 500 mg. for their reoxidation experiment, no attempt appears to have been made to determine the amount of glucose surviving such a reoxidation or to establish the presence of glucose by methods other than paper chromatography. Both of these operations would have been a considerable help towards substantiating their claim. D-glucose has, however, been confirmed by preparation of the p-nitroanilide derivative from the material after 5.5 days oxidation, and this must be considered the main basis of the claims put forward in spite of the short oxidation time.

One feature remaining, which could possibly invalidate the results obtained is the probable heterogeneous nature of the reaction. Gelatinisation of the starch at 68 - 75°C. is unlikely to give a completely dispersed solution. Waxy maize starch heated on a boiling water bath for 30 minutes still leaves a small percentage of finely divided material which can be removed by centrifugation or filtration. Starch [~~much more incompletely~~] dispersed under the conditions employed by Hamilton and Smith is liable to be rather incompletely dispersed, and periodate oxidation of the undispersed material, being very much slower than that of the dispersed material, could give completely erroneous results.

Periodate /

Periodate oxidation and residual glucose determinations have been carried out on the β-limit dextrin of the waxy maize starch and 0.33% glucose was found. The fact that β-amylolysis of the starch fails to concentrate the percentage of periodate resistant glucose units indicates that β-amylase itself can hydrolyse these anomalous linkages or that the enzyme preparation contains an impurity which can carry out this hydrolysis. This should mean that the β-amylase preparations are capable of hydrolysing a mixture of α-1,3- and α-1,4-linkages. Not only does this observation infer properties for the degradative enzyme, but it also infers, that during the synthesis of starch there is present an enzyme, as yet undetected, which is capable of synthesising these postulated 1,3-linkages yet only introduces them to the extent of one in every 200-500 linkages.

On a biochemical basis it would appear improbable that there should be any 1,3-linkages in starch, and the work of Hamilton and Smith, while adding to the number of independent groups who have found glucose after periodate oxidation of starch or its components, appears to produce inconclusive evidence that 1,3-linkages are present in waxy maize starch.

The oxidation of three samples of amylopectin (one of them a sample of waxy maize starch) and one sample of glycogen has therefore been carried out and the residual glucose contents estimated under the conditions described by Hamilton and Smith. As an aid to the evaluation of the results /

results obtained from these experiments, control oxidations have been carried out on lichenin, β-methyl maltoside and potato amylopectin and a study has been made of the effect of formic acid and sodium iodate on the viscosity of amylose and amylose polyaldehyde solutions.

EXPERIMENTAL.

Periodate Oxidation of Potato Amylopectin.

Amylopectin (10g.) dissolved in water (100ml.) filtered through a sintered disc porosity No. 3 and transferred to a 500ml. graduated flask. To this solution was added sodium metaperiodate (42.78g.) and distilled water slowly added with continuous shaking to give a homogeneous solution with a final volume of 500ml. The solution was set aside in the dark and aliquots (50ml.) were withdrawn after 4, 8, 13, 17, 20 days for the analysis of periodate uptake, formic acid release, and residual glucose content.

Periodate uptake was measured on an aliquot (1ml.) diluted with distilled water to 1 litre; 15ml. of this solution was diluted to 100ml. with distilled water and the absorbance measured against a water blank at 222.5 mμ in a Unicam S.P. 500 spectrophotometer.

Formic acid release was measured on an aliquot (4ml.) to which was added ethylene glycol (1ml.) and the solution set aside in the dark for 20 minutes. To the solution was added 10% W/v potassium iodide solution (10ml.) and the liberated iodine titrated with 0.002N sodium thiosulphate.

Residual glucose content was estimated on the remainder of the 50ml. aliquot (45ml.). To this aliquot was added ethylene glycol (4ml.) and the solution set aside in the dark for 30 minutes. The iodate in the solutions

was /

was removed by precipitation with barium acetate and filtering the solution through Whatman No. 42 paper. Potassium borohydride (2.0g.) in water (25ml.) was added slowly to the solution with stirring and the solution allowed to stand for 24 hours before destroying the excess borohydride by the addition of 6N sulphuric acid dropwise until the solution attained pH 7.0. The solution was evaporated to dryness under reduced pressure and the resultant material dissolved in 2N sulphuric acid (25ml.) before heating on a boiling water bath for 3 hours, neutralising with barium carbonate and removing the precipitate thus formed by centrifugation.

The neutralised acid hydrolysate was evaporated to a syrup under reduced pressure and dissolved in warm 80% v/v aqueous ethanol (100ml.). The crystals which separated on cooling were removed by filtration, recrystallised from hot 80% aqueous ethanol (30ml.) and filtered. The two filtrates were combined, evaporated to dryness under reduced pressure and the resulting syrup dissolved in water and made up to a final volume of 100ml. with distilled water. An aliquot of this solution (2ml.) was greatly reduced in volume, applied to a sheet of Whatman No. 1 paper along a line of 16 ins. long drawn parallel to the shorter side of the paper. Glucose control spots were applied to the paper which was developed in solvent I for 12 hours and then dried in a current of warm air /

air. The portion of the chromatogram likely to contain glucose, as indicated by spraying the control strips with aniline oxalate reagent, was cut out as a 3 inch wide strip which was eluted with distilled water in a petri dish for 30 minutes. Aliquots of this solution were treated with phenol sulphuric acid reagent (55) and the colour produced measured against a water blank in an Eel photoelectric colourimeter using a number 623 filter.

At the end of 25 days the pH of the reaction mixture was 2.9.

Time (days)	4	8	13	17	20
IO'_4 uptake (mole IO'_4/mole a.h.g.)	0.4	1.5	1.6	2.0	2.2
Formic acid release (mole HCOOH/mole a.h.g.)	-	-	0.81	0.85	0.89
Residual glucose (%)	0.64	0.54	0.20	0.08	0.11

Oxidation of Waxy Maize Starch IV at 2-4°C.

10g. of starch previously defatted and dried under vacuum over phosphorus pentoxide was gelatinised in approximately 250ml. water at 65-70°C. and the solution allowed to cool. Sodium metaperiodate (42.8g.) was added to this solution and then water was added to give a final volume of 500ml. The solution was set aside in the dark at 2-4°C. in a refrigerator and shaken daily. After 20 days it was found that the refrigerator temperature had been lowered to -10°C. and that the flask containing the experiment /

experiment had shattered. Throughout the whole of the 20 days the starch "solution" was a semi-solid jelly and a considerable mass of periodate crystals remained precipitated on the bottom of the reaction flask. In view of the obvious unsuitability of the reaction conditions for the determination of unattacked glucose residues the experiment was not repeated.

Oxidation of Waxy Maize Starch IV at 26°C.

Starch (10g.) previously defatted and dried under vacuum over phosphorus pentoxide was dissolved in approximately 400ml. water by heating on a water bath for 1 hour with continuous stirring and then cooled. A small insoluble precipitate separated out on standing and the whole solution was then passed through a 3cm. diameter sintered glass disc, porosity number 3, to remove this and any other insoluble material. Sodium metaperiodate (72.8g) was added to the filtered solution, dissolved as far as possible and water slowly added with shaking to give a homogeneous solution with a final volume of 500ml. The solution was set aside in an incubator at 26°C. in the dark for 14 days. At the end of this period the excess periodate was removed by the addition of ethylene glycol and the iodate produced precipitated by the addition of barium acetate. The barium iodate precipitate was removed by filtration through Whatman No. 1 paper. The resultant polyaldehyde /

polyaldehyde solution was neutralised with 1N. sodium hydroxide using methyl red as indicator.

Reduction of Waxy Maize Starch Polyaldehyde:-

The polyaldehyde solution was reduced in volume to approximately 100ml. under reduced pressure at 40°C. To this solution was added sodium borohydride (2g.) in water (20ml.), as small aliquots, with stirring and the solution was allowed to stand over night when a further 0.5g. borohydride was added. The solution was allowed to stand for 5 hours and then neutralised with sulphuric acid. The polyalcohol solution was deionised by electrodialysis at a current strength not exceeding 400 milliamps and then evaporated under reduced pressure to a viscous syrup.

Preliminary Acid Hydrolysis of Waxy Maize Starch Polyalcohol.

Polyalcohol (approximately 0.5g.) and sulphuric acid (3N;10ml.) were heated on a boiling water bath for 3 hours, cooled, neutralised with barium carbonate and the insoluble material removed by centrifugation. The precipitate was washed twice with distilled water and the washings combined with the original supernatant solution which was then evaporated to dryness under reduced pressure.

Chromatography of Waxy Maize Starch Polyalcohol Hydrolysate.

The hydrolysate was applied as its aqueous solution to Whatman No.1 and No.3.MM paper as spots in varying concentrations /

concentrations. The papers were developed using solvents 2 and 3, and reagents 1 and 6. Glycollic aldehyde, erythritol and an unidentified spot very near the starting line of the chromatograms were shown to be present, but in no case was even a trace of glucose found (or any spot with an R_f value approximating to that of glucose) although in some cases the hydrolysate had been so heavily loaded on to the paper that it remained incompletely dried despite prolonged heating in a current of warm air.

Oxidation of Waxy Sorghum Starch II and Potato amylopectin (var Ct. Scot) at room temperature (ca. 18°C.)

The oxidation conditions for the two polysaccharides were identical. Polysaccharide (10g.) previously defatted and dried was dissolved in 400ml. of water by heating on a water bath with constant stirring for 1 hour. The solution was cooled, filtered through a No. 3 sintered glass disc and then sodium metaperiodate (42.8g.) was added. This salt was dissolved as far as possible and then water was gradually added with continuous shaking to give a homogeneous solution with a final volume of 500ml. This solution was set aside in the dark at room temperature for 14 days. Periodate uptake on the solutions after 14 days was measured on a 5ml. aliquot of the solutions using the acid thiosulphate method of Hughes and Nevell (54). In the remaining solution the excess periodate was destroyed by the addition of ethylene glycol and the iodate produced precipitated by the addition of barium acetate solution. The solution /

solution was filtered through Whatman No. 1 paper and then neutralised using 1N. sodium hydroxide using methyl red as indicator.

Periodate uptake - Waxy Sorghum 1.4 mole IO'_4/ahg.
Potato 1.5 mole IO'_4/ahg.

Reduction of Waxy Sorghum Starch and Potato Amylopectin Polyaldehydes.

The neutral polyaldehyde solution was reduced in volume under reduced pressure to approximately 100ml. To this solution was added sodium borohydride (4.0gms.) in water (20ml.) dropwise over a period of 1 hour with stirring. The solution was allowed to stand overnight. An additional 0.5gms. sodium borohydride was added and the solution allowed to stand for a further 5 hours. The solution was neutralised with 12N. sulphuric acid.

Partial Acid Hydrolysis of Waxy Sorghum Starch and Potato Amylopectin Polyalcohols.

The neutral polyalcohol solution was acidified to pH 1.0 by the addition of 2N. sulphuric acid and then allowed to stand for 20 hours at room temperature before neutralisation with barium carbonate. The solution was centrifuged, the precipitate washed twice with distilled water, and the washings and the supernatant liquor combined. Paper chromatography of this solution using Whatman No. 1 and No. 3 MM paper, solvents 1 and 3, and spray reagents 4, 5 and 6 showed the presence of glycollic aldehyde, glycerol and erythritol but failed to reveal the presence of any glucosyl erythritol.

Total Acid Hydrolysis of Waxy Sorghum Starch, Potato Amylopectin and Waxy Maize Starch (Polyalcohols).

The partial acid hydrolysate solution was adjusted in volume to 100ml. and sufficient 12N. sulphuric acid was added (35ml.) to give an apparent final sulphuric acid concentration of 3 N. The solution was heated on a boiling water bath for 3 hours, neutralised by the addition of barium carbonate, centrifuged and finally evaporated to dryness. Paper chromatography using Whatman No. 3 MM paper, solvents 1 and 2, and spray reagents 1 and 2 failed to reveal the presence of glucose.

Glucose Content of Waxy Sorghum Starch Polyalcohol.

The polyalcohol total hydrolysate was dissolved in water (80ml.) and an aliquot (0.10ml.) was applied along a line 14" long drawn parallel to the shorter side of a sheet of Whatman No. 1 paper. Glucose control spots were applied to the paper which was placed in a tank containing butanol, pyridine, water (6.4.3.), allowed to stabilise for 10 hours before developing with the solvent for 12 hours. The paper was dried at room temperature. The control strips were cut out and the position of the glucose spots revealed using the alkaline silver nitrate reagent. The portion of the chromatogram likely to contain glucose as indicated by the control spots was cut out as a 3 in. wide strip which was eluted with 20ml. distilled water in a petri dish for 30 minutes. Aliquots of this solution were

treated /

treated with phenol sulphuric acid reagent and the colour produced measured against a water blank in an Eel photo-electric colourimeter using a number 623 filter.

	Reading	Apparent Glucose µg/ml.
Control	0.16	2.4
Polyalcohol	0.15	2.2

There thus appeared to be no residual glucose after the periodate oxidation of waxy sorghum starch.

Glucose Content of Potato Amylopectin and Waxy Maize Starch Polyalcohols.

The polyalcohol total hydrolysate was dissolved in water and made up to a final volume of 100ml. An aliquot of this solution (0.2ml.) was applied to a line 14 ins. long drawn parallel to the shorter side of a sheet of Whatman No.1 paper, eluted and estimated as described for waxy sorghum starch polyalcohol.

A blank sheet of Whatman No. 1 paper was subjected to exactly the same procedure.

	Reading	Apparent Glucose µg/ml.
Waxy Maize Starch	0.16	2.4
Potato Amylopectin	0.19	2.9
Blank	0.17	2.6

Residual glucose in Potato Amylopectin Polyalcohol

$$= \frac{(2.9 - 2.6) \times 20 \times 100 \times 100}{0.2 \times 10^6 \times 10}$$

$$= 0.03\%$$

Residual glucose in
Waxy Maize Starch Polyalcohol = 0.00%

Periodate Oxidation of Oyster Glycogen (Dr. Z.H. Gunja).

Glycogen (ca. 300mg.) was dissolved in 100ml. of 5% potassium chloride solution in a dark bottle. To this solution was added 20ml. of 8% sodium periodate and the tightly stoppered bottle placed on rollers for 14 days when the excess periodate was destroyed by the addition of 5ml. ethylene glycol and after 30 minutes in the dark the solution was dialysed and freeze dried.

Reduction of Glycogen Polyaldehyde.

Polyaldehyde (0.5g.) was dissolved in water (20ml.) and sodium borohydride (1g.) in water (5ml.) was slowly added with stirring. The solution was allowed to stand for 30 hours before neutralising with 12N. sulphuric acid, and then evaporating to dryness under reduced pressure.

Total Acid Hydrolysis of Glycogen Polyalcohol.

The glycogen polyalcohol was dissolved in 3N. sulphuric acid (50ml.), heated on a boiling water bath for 4 hours, neutralised with barium carbonate, centrifuged, and finally evaporated to dryness under reduced pressure to give a viscous syrup. Paper chromatography of this syrup in solvents 1, 2 and 3 using spray reagents 1 and 2 showed the presence of a trace of glucose.

Glucose Content of Glycogen Polyalcohol.

The polyalcohol was dissolved in water (5ml.) An aliquot (0.089ml.) was applied by means of a micro pipette to a line 16 ins. long drawn parallel to the shorter side of /

of a sheet of Whatman No. 1 paper, eluted and estimated as described previously.

	Reading	Apparent Glucose µg/ml.
Control	0.16	2.4
Polyalcohol	0.21	3.2

Residual glucose in glycogen polyalcohol = 0.16%.

Preparation of Lichenin Polyalcohol.

A sample of lichenin (10g.) isolated by Dr. F.B. Anderson (56) and sodium metopaeriodate (30g.) were added to water (500ml.) and the whole shaken for 100 hours in the dark at room temperature. At the end of this period the solution was centrifuged and the supernatant liquor discarded. The precipitate was washed with distilled water to remove all ionic material, suspended in distilled water (200ml.) then treated with potassium borohydride (2.0g.) in water (50ml.) which was added dropwise over a period of one hour with continuous stirring. The reaction mixture was allowed to stand for 24 hours when an additional 0.5g. borohydride was added and the solution allowed to stand for a further 5 hours. Glacial acetic acid was added dropwise until the solution attained pH 7.0.

The removal of the ions in the solution was accomplished by electrodialysis at a current not exceeding 0.4 amp. and the resulting liquid was freeze dried to give a pure white solid. Yield 7 g.

Glucose /

Glucose Content of Lichenin Polyalcohol. (i)

Polyalcohol (91.1mg.) was dissolved in water (20ml.). Aliquots of this solution (1ml.) were subjected to acid hydrolysis using 6N. sulphuric acid (0.5ml.) and heating on a boiling water bath for 2 hours. The resultant solution was neutralised and the glucose content estimated in duplicate with Somogyi reagent.

% Glucose = 32.6, 31.8.

Glucose Content of Lichenin Polyalcohol.(ii)

Polyalcohol (150 mg.) was dissolved in 3N. sulphuric acid (20ml.) and the solution heated on a boiling water bath for 2 hours. The solution was cooled, neutralised with barium carbonate, centrifuged, and the supernatant liquid transferred to a 100 ml. graduated flask. The precipitate was washed with distilled water and the washings transferred to the graduated flask which was then made up to the mark with ethanol. An aliquot of the solution (0.8ml.) was applied to a line 14 ins. long drawn parallel to the shorter side of a sheet of Whatman No. 1 paper; glucose control spots were applied to the paper which was then developed with solvent 2 for 12 hours after allowing the paper to equilibrate with the solvent vapour for 10 hours. The glucose containing band was eluted and the glucose estimated as described perviously.

	Reading	Glucose (μg/ml.)
Lichenin Polyalcohol	1.20	20.5
Blank	0.17	2.6

$\therefore$ Residual Glucose in Lichenin Polyalcohol = 26.9%

Periodate Oxidation of Lichenin.

Lichenin (1.0gm.) was dissolved in water (25ml.) with the aid of heat. The solution was cooled and sodium metaperiodate (4.278gm.) was added. Distilled water was slowly added to the reaction mixture, with continuous shaking, to give a solution with a final volume of 50ml. containing no undissolved periodate. The solution was set aside in the dark and samples were withdrawn after 4, 8, 12, 16 and 20 days for the analysis of periodate uptake, formic acid release, and residual glucose.

0.5Ml. of the solution was removed for measurement of periodate uptake, diluted to 500ml. with distilled water, and 15ml. of this solution further diluted to 100ml. with distilled water. The absorbance of this solution was measured at 222.5mμ against a water blank in a Unicam S.P. 500 spectrophotometer and the periodate uptake calculated as per Aspinall and Ferrier (33).

1.0Ml. of the solution was removed for determination of formic acid release, diluted with distilled water (10ml.), ethylene glycol (1ml.) added, and the solution left in the dark for 20 minutes. Freshly prepared, neutral, 10%^{W}/v potassium iodide solution was added and the liberated iodine titrated with 0.01N sodium thiosulphate.

2.5Ml. of the solution was removed for the estimation of residual glucose content, diluted with distilled water /

water (10ml.) and ethylene glycol (1ml.) added to the solution which was then set aside in the dark for 20 minutes before neutralising with barium carbonate and precipitating the iodate with barium acetate. The precipitate was removed by filtration and the solution reduced by the addition of sodium borohydride (0.5g.) and then allowing the solution to stand for 24 hours before destroying excess borohydride by the dropwise addition of 6N sulphuric acid until pH 7.0 was attained. Sufficient 12N sulphuric acid was added to make the solution 3N with respect to sulphuric acid concentration and the solution heated on a boiling water bath for 2 hours. At the end of this time the solution was cooled, neutralised by the addition of barium carbonate and the precipitate removed by centrifugation. The precipitate was washed with distilled water and the washings combined with the supernatant liquor from the centrifugation in a 100ml. graduated flask and the volume made up to the mark with ethanol. 0.2Ml. of this solution was applied to a sheet of Whatman No. 1 paper and the glucose content estimated as described previously.

Time (days)	4	8	12	16	20	24
IO'_4 uptake (mole IO_4/mole ahg.)	1.12	1.35	1.75	2.03	2.10	2.29
HCOOH release (mole HCOOH/mole ahg.)	0.41	0.52	0.71	0.82	1.02	1.13
Residual glucose (%)	-	28	22	-	16	14

Periodate Oxidation of β-Methyl Maltoside.

β-Methyl maltoside (15.2mg.) (kindly provided by Dr. K. Hunt) was dissolved in 0.40M sodium /

sodium periodate (10ml.) and set aside in the dark at room temperature.

Aliquots (1.0ml.) were withdrawn for the measurement of formic acid release, diluted with water (10ml.) before adding ethylene glycol (1ml.) and setting aside in the dark for 20 minutes. At the end of this time freshly prepared neutral 10%^{W}/v potassium iodide solution (1ml.) was added and the liberated iodine titrated with 0.001N sodium thiosulphate.

Time (days)	4	8	12	16	24
HCOOH release (moles HCOOH/mole β-m-m)	1.03	1.17	1.17	1.28	1.57

Stability of amylose at pH 2.9.

Amylose (approx. 2g.) was dissolved in water (100ml.) with heating. Sodium iodate (2.5gm.) was added to the solution and then 90% formic acid dropwise until the pH attained a value of 2.9. The solution was filtered through a sintered glass filter porosity No. 3 into a modified Ubbelhode viscometer maintained at 25^{o}C in a constant temperature water bath. The flow times were measured at 0, 10, 20 days.

Time (days)	0	10	20
Flow time (secs.)	150.26	150.28	151.56

Stability of amylose polyaldehyde at pH 2.9.

Amylose (1g.) was dissolved in a solution of sodium metaperiodate (100ml., 4M) with shaking and then set aside /

aside in the dark at room temperature for 50 hours. The excess periodate was destroyed by the addition of ethylene glycol and the solution dialysed against running tap water for 48 hours. The solution was evaporated to dryness under reduced pressure at 35°C, dissolved with shaking in a saturated solution of sodium iodate (20ml.) which had been adjusted to pH 2.9 by the dropwise addition of 90% formic acid. This solution was filtered through a No. 3 glass sinter into a modified Ubbelhode viscometer and the flow time noted after various time intervals. The flow time of the sodium iodate/formic acid solution was 618.21 seconds.

Time (days)	0	2	4	6	8
Flow time (sec.)	697.56	696.91	697.51	697.58	697.50

Average Chain Length (CL) of Oyster Glycogen, Waxy Maize and Waxy Sorghum Starch, and Potato amylopectin by Potassium metaperiodate oxidation.

Polysaccharide (350mg.) previously dried under vacuum over phosphorus pentoxide was dissolved in 5% potassium chloride solution (100ml.). An aliquot of this solution (80ml.) and 8% sodium periodate (20ml.) were pipetted into a dark glass bottle which was tightly stoppered and kept continuously agitated. Aliquots of the reaction mixture (15ml.) were removed after 200 and 300 hours, ethylene glycol (3ml.) was added and the solution kept in the dark for 20 minutes before estimating the formic acid release either by titration with 0.01N sodium hydroxide to pH /

pH 5.8 under an atmosphere of nitrogen or by adding excess 10% potassium iodide solution (5ml.) and titrating the liberated iodine with 0.01N sodium thiosulphate.

	C L	
Time (hours)	200	300
Waxy maize starch	23.5	24.0
Waxy sorghum starch	22.5	22.5 (at 250 hours).
Potato Amylopectin	24.0	24.0
Oyster Glycogen	10.5	10.5

Average Chain Length (CL) of Waxy Maize and Waxy Sorghum Starches, Potato Amylopectin and Oyster Glycogen by Sodium Metaperiodate oxidation at 2°C (80).

Polysaccharide (ca. 200mg.) was dissolved in water (50ml.) and the solution cooled to 2°C. Sodium meta-periodate solution (8.0% W/v; 30ml.) was added and the solution made up to 100ml. with distilled water and then cooled to 2°C. Aliquots (20ml.) were removed after 15, 35 and 60 hours, the excess periodate destroyed by the addition of ethylene glycol (1ml.) and storing in the dark for 20 minutes. Potassium iodide solution (10% W/v; 3ml.) was added and the solutions titrated with 0.005N sodium thiosulphate.

	C L		
Time (hours)	15	35	60
Waxy Maize Starch	24.7	24.7	22.0
Waxy Sorghum Starch	22.6	22.6	19.8
Potato Amylopectin	26.4	22.9	19.9
Oyster Glycogen	11.3	10.4	9.8

β-amylosis /

β-amylolysis limits of Waxy Maize Starch and Waxy Sorghum Starch at pH 4.6.

To a 50ml. graduated flask was added enzyme solution (10ml.;200 units/ml.), polysaccharide solution (30ml.; 1.6mg./ml.) acetate buffer (5ml.; pH 4.6; 0.2M.) and the solution made up to the mark. Aliquots (4ml.) were removed and the maltose content estimated by the Somogyi reagent.

	Time (hours)	β-limit (%)
Waxy Maize Starch	26	50
	58	58
	82	57
Waxy Sorghum Starch	48	55
	72	59
	120	58

β-Amylolysis limit of Potato Amylopectin and Oyster Glycogen at pH 4.6

To a 50ml. graduated flask was added polysaccharide solution (30ml.; 1.6 mg./ml.), enzyme solution (2.5ml.; 200 units/ml.), "digest base" (5ml.) and the solution made up to the mark with distilled water. Aliquots (4ml.) were removed for the estimation of maltose content using the Shaffer Hartmann reagent.

	Time (hours)	β-limit (%)
Potato Amylopectin	3	50
	25	53
	50	55
	75	54
Oyster Glycogen	3	33
	25	40
	50	43
	75	43

TABLE 2.

Properties of Amylopectins and Glycogens.

Polysaccharide	CL.	ICL.	β-limit	Notes
Waxy Maize Starch	20	2-3	-	Hamilton & Smith
Waxy Maize Starch IV	24	6-7	58	This work
Waxy Sorghum Starch	22	5-6	58	"
Potato Amylopectin	24	7	55	"
Oyster Glycogen	10	2-3	43	"
Amylopectins:-				
Potato I	23	7-8	52	(58)
Potato III	22	5	53	"
Waxy Maize I	22	6-7	53	"
Protozoal	22	5-6	63	"
Glycogens:-				
Human Muscle	11	3	41	"
Ox Muscle	15	4	50	"
Rabbit Liver IV	13	3-4	45	"
Cat Liver IV	13	2-3	53	"
Skate Liver	13	3-4	45	"

Discussion.

In order to draw a conclusion relating to the fine structure of a starch-type polysaccharide it must first be shown that the sample used has properties which can be regarded as "normal" for the polysaccharide. Thus the four samples used in this work show normal values for both chain length and β-amylolysis limit (see Table 2). This is in contrast to the sample of waxy maize starch used by Hamilton and Smith in their experiments which, though having a normal chain length, produced a β-limit dextrin with a chain length of only 6, indicating an interior chain length (ICL.)of 2-3 differing markedly from the more usual values of between 5 and 8.

An attempt to oxidise a sample of waxy maize starch under the low temperature (2-4°C) condition of Hamilton and Smith has shown that the results obtained by these workers at this temperature are probably invalid. It is impossible to prepare 0.4M sodium periodate solution at 2-4°C., crystals of the periodate crystallise out to form a solid mass on the bottom of the reaction flask, and it is doubtful if periodate uptakes can be measured with any accuracy under these conditions. The polysaccharide solution, although completely dispersed, in contrast to the suggested gelatinisation at 68 - 75°C, set to a jelly on cooling and vigorous shaking of the reaction flask, at intervals, throughout the 19 days for which the 2-4°C temperature was maintained, only broke this mass up into

small /

small pieces which showed no sign of dissolving. These conditions will allow only a slight transfer of periodate from the precipitated material throughout the reaction mixture and this, together with the heterogeneous nature of the reaction, makes this method unsuitable for the determination of fine structure.

The oxidation conditions at about 20°C, however, are much more suited to the nature of the investigation in that, provided the sample is adequately dispersed, they suffer from none of the disadvantages described above. An investigation on the effect of the oxidation time on the percentage of residual glucose has been carried out at room temperature (18 - 20°C.) on a sample of potato amylopectin. The results obtained indicate that there is no level value which could in any way be ascribed to resistant glucose units although the periodate consumption and the formic acid release are both much in excess of that required for a "Malapradian" oxidation. After 24 days the pH of the oxidation mixture was 2.9.

Three samples of amylopectin have been oxidised with sodium periodate for 14 days. These samples were dispersed at 100°C. and then filtered through a sintered glass disc to ensure complete homogeneity of the reaction mixture. A sample of a glycogen polyaldehyde isolated at the end of a chain length determination, using sodium periodate in the presence of potassium chloride (57), has also been examined for residual glucose content. The results obtained /

obtained are shown in Table 3 below.

TABLE 3.

Residual Glucose Contents of Amylopectins and a Glycogen.

Polysaccharide	Oxidation Temp. (°C.)	Time (days)	% Glucose
Waxy Maize Starch	26	14	0.00
Waxy Sorghum Starch	18-20	14	0.00
Potato Amylopectin	18-20	14	0.03
Oyster Glycogen	18-20	14	0.16

The glycogen sample yielded a small percentage of residual glucose but no attempt had been made to completely oxidise the sample and it can thus be said that the glycogen sample contains less than 0.16% of periodate resistant glucose units.

From the results obtained, which are in contrast to those of Hamilton and Smith, the simple conclusion is that there are no 1,3-linked or 1,2,4- or 1,3,4-linked glucose units in amylopectin. The periodate oxidation conditions for the amylopectins are, however, rather severe and the possibility that the occurrence of over-oxidation would eliminate even 1,3-linked glucose residues could not be ignored. A control experiment with lichenin, a β-1,3- and β-1,4-linked polysaccharide, using 0.4M sodium periodate at room temperature has shown that the content of residual glucose gradually decreased with time. The percentage of periodate resistant β-1,3-linkages in this polymer was 27 as measured by the phenol sulphuric acid technique /

technique, and 32 by cuprimetric estimation. This latter value may be slightly high since glycollic aldehyde, which interferes with this method of estimation, may not be completely destroyed during the acid hydrolysis. At the end of 24 days the residual glucose content was 14%.

The overoxidation of lichenin may occur in two ways. (1) There may be a random breakdown of the polysaccharide chain so that 1,3-linked glucose residues become end groups and therefore susceptible to attack by periodate. Or (2) Stepwise oxidation from the reducing end group (59, 60).

Both amylose and amylose polyaldehyde in the presence of sodium iodate and formic acid at pH 2.9 show no appreciable change in viscosity. Thus random breakdown is not the source of the overoxidation of lichenin which must therefore be attributed to the stepwise process from the reducing end. This latter, however, cannot occur in amylopectin where the 1,6-linkages act as barriers to the process.

Oxidation of β-methyl maltoside with 0.4M sodium periodate has shown that overoxidation occurs after 2 days. In this case there is also no possibility of overoxidation from a reducing end group.

Applying the results of these control experiments to amylopectins it would appear that the high formic acid production, similar to that reported by Hamilton and Smith, and noted by Perlin (61) for the oxidation of glycogens, arises /

arises from oxidation of dialdehyde groups and not from random degradation (62). The proportion of the periodate resistant glucose units is therefore not going to be decreased to any appreciable extent by overoxidation.

The conclusion which must be drawn from this work is that provided amylopectin is adequately dispersed and the periodate oxidation is taken to completion there are no periodate resistant linkages in amylopectin.

SECTION 3.

PART 2.

Evidence for 1,3-linkages in starch from acid reversion studies.

INTRODUCTION.

The acid catalysed reversion of sugars was first studied with respect to the synthesis of large polymers (63, 64). It has more recently attracted attention as a factor to be considered in the linkage analysis of polysaccharides, by acid hydrolysis, in that it may give rise to erroneous conclusions as to the structure of the polysaccharide under consideration.

Glucose, in the presence of acid, is known to undergo a series of reactions which produce inter- and intra- molecular condensation products. The principle inter- molecular products are disaccharides. If it is assumed that glucose, which exists largely in the pyranose form in solution, reacts as such, then there are eleven possible disaccharides which could be produced. Seven of these disaccharides have in fact been isolated in varying amounts from a glucose reversion mixture by Wolfrom and his co-workers (65), and characterised as their octa-acetates. By far the greatest amount of disaccharide results from the condensation of the hemiacetal group with the primary hydroxyl group on C_6 giving almost equal quantities of isomaltose and gentiobiose. Intramolecular condensation has

been /

been shown to give rise to laevo-glucosan (1,6-anhydro-β-D-glucose) of which both the furanose and pyranose forms have been detected (66), while furfuraldehyde, which may be regarded as a glucose decomposition product, has also been detected in quantity (67).

TABLE 4.

Isolation of isomaltose from reversion mixtures.

Initial sugar concentration (%)	Acid concentration (N.)	Time of heating (hours)	Iso-maltose (mg.)	Refer-ence.
33	0.08	10	2100	(65)
10	0.08	10	250	(68)
2	0.06	24	600	(70)
2	0.06	8	375	(70)
1	0.33	2	52	(66)
1	0.33	5	74	(66)
1	0.33	10	105	(66)

Yield of isomaltose calculated per 100g. initial sugar.

Of the many reversion experiments carried out on glucose (See Table 4.) in only two has any attempt been made to give some insight into the factors involved in the reaction. Thompson, Wolfrom, and Quinn (68) have shown that the production of isomaltose and gentiobiose on heating solutions of glucose in 0.083 N. hydrochloric acid for 10 hours at 100°C. depends directly on the initial glucose concentration in the range 0.4 - 10.0% ($^{W}/_{v}$). Peat and his co-workers (66) on the other hand, demonstrated, with 1% $^{W}/_{v}$ glucose solutions in 0.33 N. sulphuric acid at 100°C., that the /

the production of disaccharides by reversion is not proportional to the time the reaction proceeds, but, that considerably more disaccharide is formed in the first two hours than from the second to the fifth or the fifth to the tenth hours.

The partial acid hydrolysis of amylopectin and glycogen has been investigated in detail, and the presence of α-1,6-linkages in these polysaccharides has been shown by the isolation of isomaltose (6-O-α-D-glucopyranosyl-D-glucose). Control experiments have shown that, although isomaltose is the main disaccharide arising from acid reversion, the amounts obtained were in excess of reversion yields. Wolfrom (68) has shown that the isomaltose from a partial hydrolysate of waxy maize starch was in 200 fold excess of reversion, while Bacon and Bacon (70) obtained an 8 fold excess for the isomaltose from a hydrolysate of glycogen. The percentage of 1,6-linkages known to occur in glycogen is higher than that in amylopectin, and the difference in the quoted excesses indicates the care needed when suggesting minor structural features from partial acid hydrolysis experiments. The presence of 1,6-linkages has however been confirmed by the isolation of a trisaccharide containing an α-1,6-linkage, and by enzymic studies.

A structural feature of amylopectin and glycogen which has received support from acid hydrolysis, but not as yet from enzymic studies, is the occurrence of a very small percentage /

percentage of α-1,3-linkages in these polymers. The evidence for these linkages from periodate oxidation studies has been dealt with in Part I. of this section. The possible occurrence of this linkage arises from the isolation of nigerose (3-O-α-D-glucopyranosyl-D-glucose) from the partial acid hydrolysates of amylopectin (69), Floridean starch (71), and glycogen (72) in amounts stated to be in excess of that produced by reversion.

Nigerose was first isolated from a partial acid hydrolysate of the fungal polysaccharide nigeran by Barker, Bourne and Stacey in 1953 (73), although it had apparently been chemically synthesised by Gakhokidze in 1946 (74). Its structure has been established by methylation (75), by the fact that it forms the same osazone as turanose (3-O-α-D-glucopyranosyl-D-fructose), and by chemical synthesis (76). Nigerose may be characterised by its specific rotation in water ($[\alpha]_D$ + 135-137°), and the melting points of its octaacetate (149-152°C.) and its osazone (204-206°C.). All the samples so far isolated, with two exceptions, agree with these values. Both Pazur (77) and Gakhokidze (74) have obtained low values (+87° and +85° respectively) for the specific rotation of the disaccharide. Gakhokidze's preparation was probably impure, but, with that of Pazur, obtained by chromatography from a reversion mixture, it is difficult to account for the low rotation. Pazur's work is of interest, not only for the anomalous rotation of his nigerose /

nigerose, but also in that he isolated 400mg. of the disaccharide from a mixture of 12 g. glucose and 24 g. maltose heated in 90ml. of 0.1 N. hydrochloric acid for 5 hours at 100°C. (a 1% yield).

Nigerose has been isolated from reversion mixtures by two other groups of workers. From the results obtained (See Table 5) it can be stated that the yield of the disaccharide increases with time and also with increasing sugar concentration.

TABLE 5.

Isolation of nigerose from reversion mixtures.

Initial sugar concentration (%)	Acid concentration (N.)	Time (hours)	Nigerose (mg.)	Reference.
40	0.1	5	1100	(77)
33	0.08	10	110	(65)
1	0.33	2	14	(71)
1	0.33	5	15	(71)
1	0.33	10	25	(71)

Yield of nigerose calculated per 100gm. initial sugar.

Thompson and Wolfrom have detected 2mg. nigerose octaacetate after acetylation of a partial acid hydrolysate of 96g. of glycogen (72) and have attached structural significance to this finding. It seems probable in this case that the nigerose is a reversion artefact with no relevance to the structure of the glycogen. The yield is only 0.001%. These same authors have isolated nigerose (175mg.) from a partial acid hydrolysate of amylopectin (130g.) (69). On the /

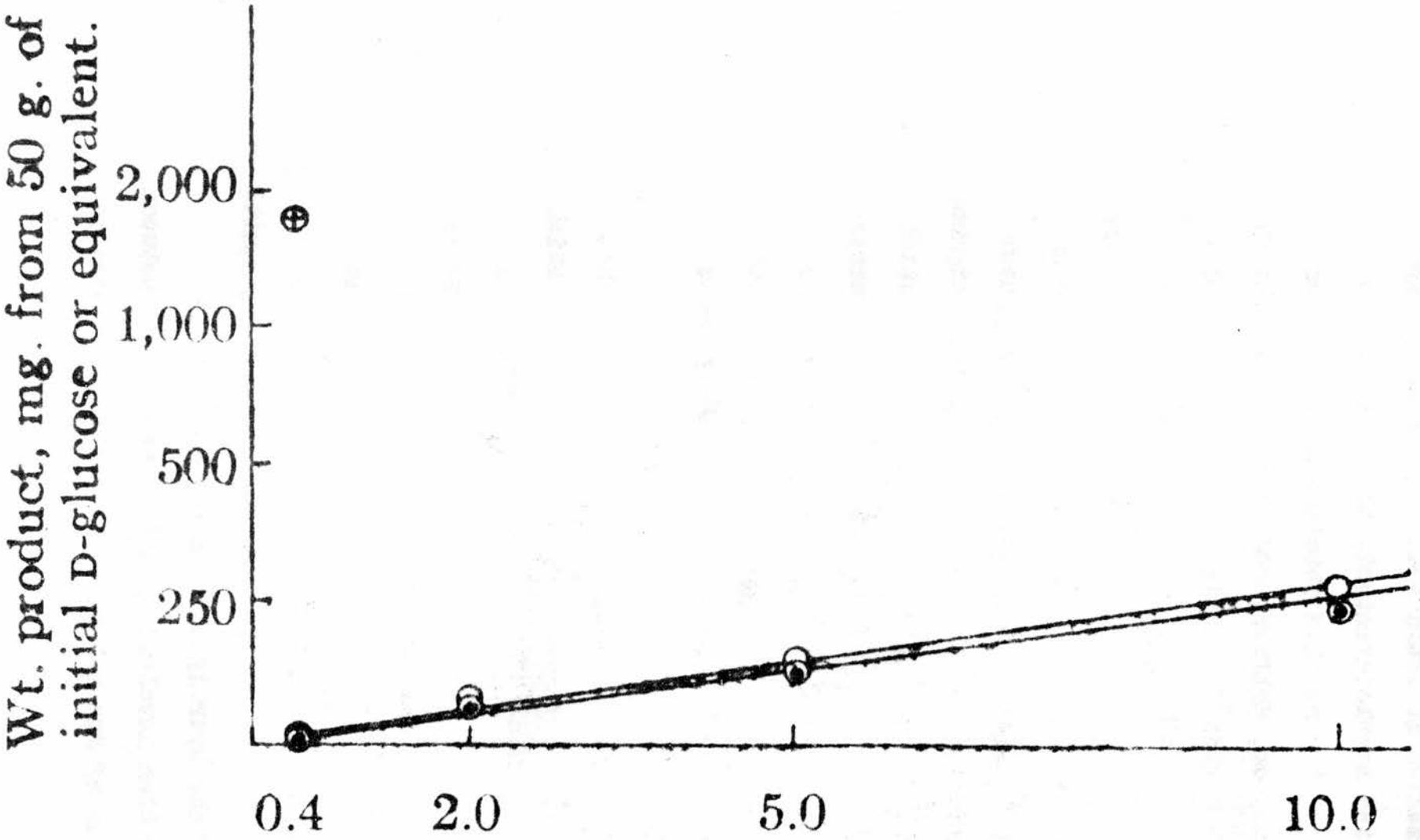


Fig. 1.—Yields of products obtained on hydrolysis for 10 hr. with 0.082 *N* HCl at 97° at varying initial reactant concentration; β-isomaltose octaacetate from amylopectin (⊕) and from D-glucose (○); β-gentiobiose octaacetate from

the evidence presented it would seem that α-1,3-linkages were present in the sample examined, though it may be possible to criticise the results obtained in the control experiment of Thompson, Wolfrom, and Quinn (68). The "y" axis of the graph containing their data (see opposite) is apparently on a logarithmic scale to the base 2 and its intersection with the "x" axis should be "125mg." not "0mg." as might readily be supposed. Unfortunately a table of the actual results obtained is not included in the paper.

Peat, Turvey and Evans (71) isolated 34mg. nigerose from 12.5g. of Floridean starch under conditions in which only 2mg. would have been formed from glucose by reversion. A trisaccharide fraction containing α-1,3-linkages was also isolated. On β-amylolysis a disaccharide was detected which had the same chromatographic and electrophoretic mobility as nigerose.

There are thus two reported occurrences of the isolation of nigerose from partial acid hydrolysates which may indicate the possible presence of α-1,3-linkages in amylopectin. Both amounts of disaccharide are in excess of that produced by glucose reversion under the same conditions and the main barrier to their acceptance as being structurally significant is whether the results obtained from the reversion of a monosaccharide are a suitable control for the hydrolysis of a polysaccharide.

The reaction involved in acid reversion is between the reducing group of one molecule and a hydroxyl group on

a /

a second molecule with the elimination of the elements of water. During the hydrolysis of a polysaccharide the concentration of the reducing groups in the solution will obviously be less than when an equivalent amount of glucose is treated with acid, and it would not be unreasonable to expect that the amounts of disaccharides formed from reversion during the hydrolysis of a polysaccharide would be less than that formed from glucose.

There are, however, two other factors which may operate in the opposite direction. Firstly, as noted by Peat and his co-workers (71), the rupture of a glycosidic bond by acid will produce two fragments which may combine with the elements of water, with each other, or with other fragments, and, in this latter case the energy associated with the original bond would be available for the formation of the new linkage. Secondly, immediately on hydrolysis of a glycosidic bond the fragments are very close to each other and may re-combine in the same or in another position. This type of re-combination cannot occur with a glucose control.

An element of doubt therefore exists as to the suitability of glucose as a reversion control. It would seem more suitable to use at least a disaccharide having the same linkage as the main chain-forming linkage in the polymer. This would appear to be particularly necessary in view of Pazur's finding (77) that a mixture of glucose and maltose is more suitable for the production of nigerose by reversion than glucose alone. Comparison of the

yields /

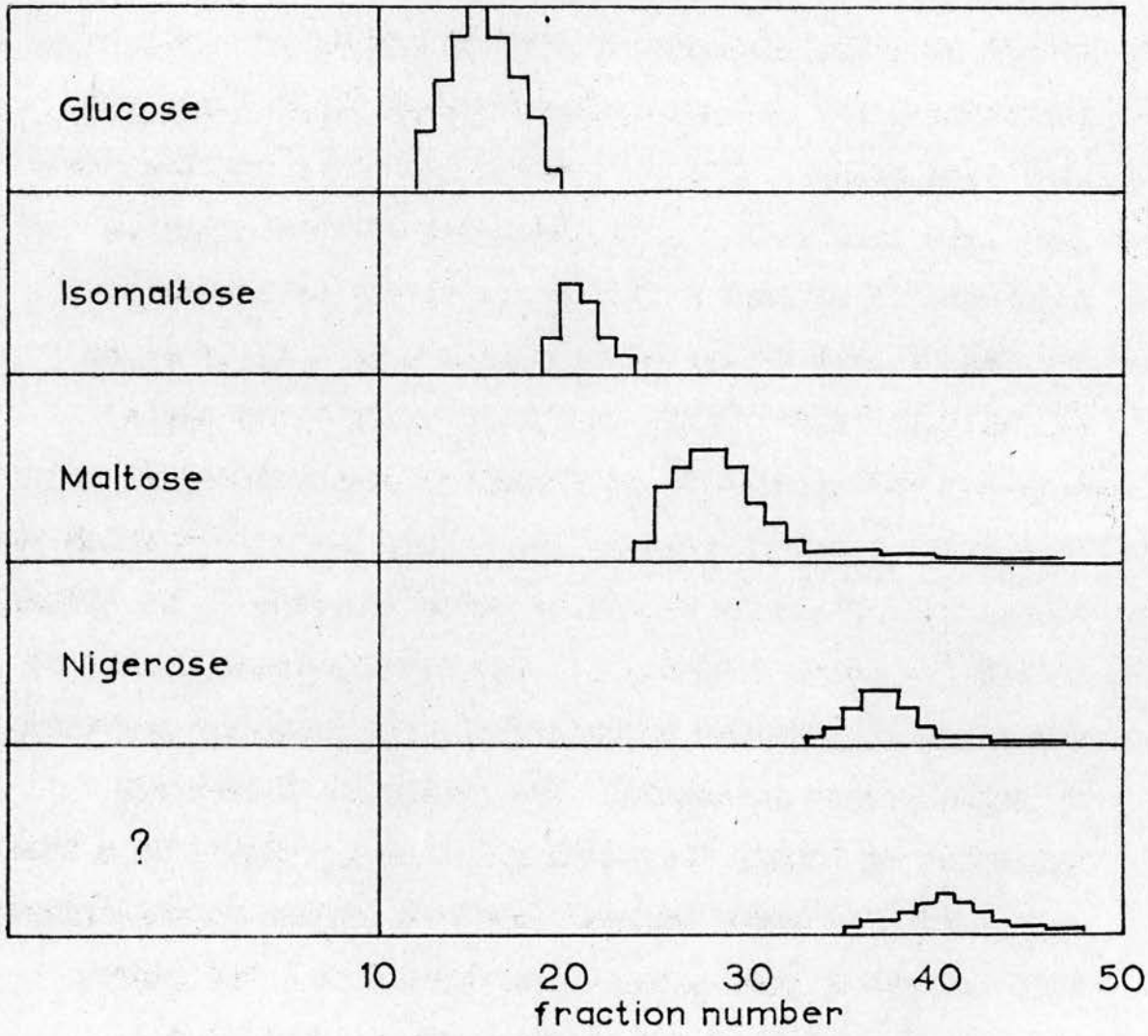
yields of nigerose isolated by Pazur with the glucose - maltose mixture and those of Wolfrom (68) under rather similar conditions (see Table 4) would seem to indicate that the effect may be greater than indicated by Pazur.

Some direct comparison between the reversion products obtained from maltose and from glucose under the same conditions is obviously desirable in order to determine the usefulness of glucose as a reversion control. This has been carried out using the identical conditions of Peat and his co-workers (66) and as used by Peat, Turvey and Evans (71) for the hydrolysis of Floridean starch. In view of the isolation, by these latter workers, of a small quantity of material tentatively identified as nigerose, from the β-amylolysis of Floridean starch, a sample of this starch has been subjected to enzymic hydrolysis and the resultant material examined for the presence of nigerose.

The work of Pazur and Budovitch (77) has been repeated in an attempt to resolve the anomalous rotation and to confirm, if possible, the high yield of nigerose obtained by them.

The use of C^{14} -Glucose in the study of reversion has not yet been reported although it has found use in enzymic studies (78,79). An attempt has been made to compare the products of acid reversion from glucose, maltose and amylopectin using generally labelled C^{14}-Glucose.

Figure 6 Glucose + Maltose reversion charcoal column



EXPERIMENTAL.

1. Acid reversion of a mixture of glucose and maltose.

A mixture of glucose (12g.) and maltose (24g.) was dissolved in sulphuric acid (90ml.; 0.1N.) and heated on a boiling water bath for 5 hours. The solution was cooled rapidly, neutralised with excess barium carbonate, centrifuged and the precipitate washed three times with distilled water. The supernatant solution and the washings were combined. A chromatogram of this solution developed in solvent 2 (two ascents) and sprayed with aniline oxalate revealed the presence of 5 spots of R_f values 0.61, 0.50, 0.45, 0.37, and 0.25. The whole solution was applied to an Ultrasorb charcoal-Celite column (100 x 6cm.) and allowed to drain into the column which was eluted with 5 litres distilled water followed by 25 litres of 10% $^{v}/_{v}$ aqueous ethanol. The total aqueous eluate on reduction to a volume of 1ml. failed to show the presence of carbohydrate material. The ethanolic eluate was collected in 500ml. fractions which were reduced to a final volume of 1ml. under reduced pressure before chromatography with solvent 2 (two ascents) on Whatman No. 3MM paper. The results obtained are shown opposite (Figure 6). Fractions were combined as follows:-

11 - 19	(O)
20 - 24	(A)
25 - 32	(B)
33 - 50	(C)

Fraction O /

Fraction O 26.1g. dry weight was reduced in volume under reduced pressure to a thick syrup which crystallised on standing. Chromatography using solvents 1 and 2 and aniline oxalate as the spray reagent revealed the presence of only one spot having the same chromatographic mobility as glucose. Acetylation of the sugar gave an acetate which, after two recrystallisations from ethanol, had a melting point of 130 - 131°C. The specific rotation of the sugar was $[\alpha]_D$ + 52° in water. Values quoted for glucose are:- $[\alpha]_D$ + 52.6° and β-pentaacetate m.p. 131°C.

Fraction A was evaporated to dryness under reduced pressure. Chromatography using solvent 2 and aniline oxalate as the spray reagent showed the presence of three spots two of which were in trace amounts. By comparison with controls the fraction was shown to contain traces of glucose and maltose, and a considerable amount of isomaltose. The isomaltose was isolated free from the traces of glucose and maltose by descending chromatography using solvent 1 on sheets of Whatman No. 3MM paper and eluting the appropriate bonds. This gave 195.2mg. (dry weight) of a sugar which was chromatographically homogeneous. Acetylation of a portion of this fraction gave a crystalline product melting point 142°C. after one recrystallisation from ethanol. The specific rotation of the sugar was +122.8° in water. Quoted values for isomaltose are:- $[\alpha]_D$ + 122° in water and β-octaacetate m.p. 143°C.

Fraction B /

Fraction B was evaporated to dryness under reduced pressure to give 3.15g. crystalline material. Chromatography using solvent 2 and aniline oxalate as the spray reagent showed the presence of two spots having the same chromatographic mobility as glucose and maltose. A portion of the material was applied to a sheet of Whatman No. 3MM paper and developed with solvent 2. The appropriate bands, as revealed by spraying marker strips with alkaline silver nitrate, were eluted with distilled water and evaporated to dryness in tared flasks giving 33.7mg. glucose and 303mg. maltose. The specific rotation of the maltose was 132.9° in water and acetylation gave a syrup which after two recrystallisations from ethanol gave crystals melting point 158°C. Quoted values for maltose are $[\alpha]_D$ + 136° , octaacetate m.p. 158 - 159°C. Fraction B thus contained 315mg. glucose and 2835mg. maltose.

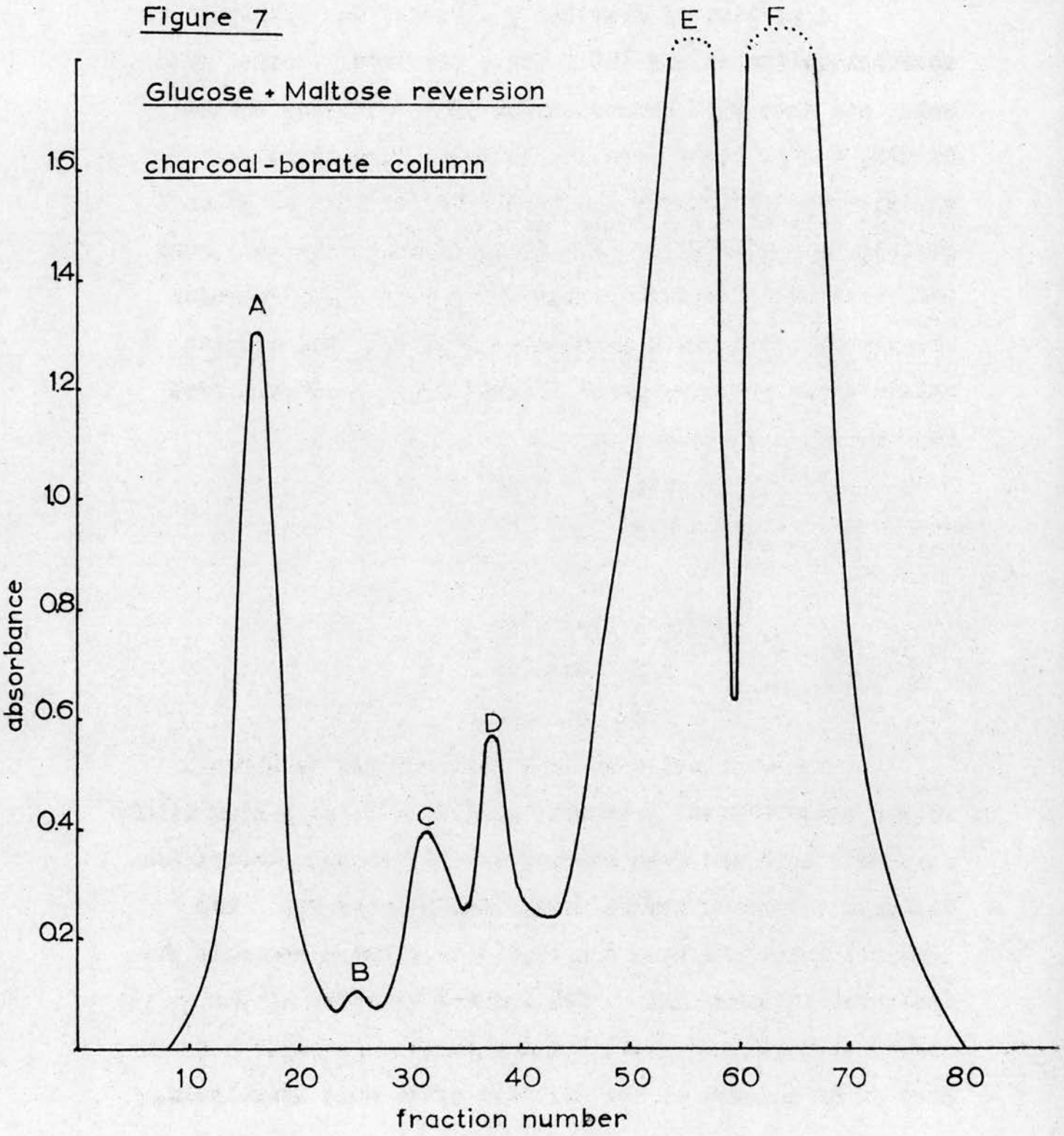
Fraction C was evaporated to dryness under reduced pressure to give 1.98g. material which on chromatography with solvent 2 (three ascents) and aniline oxalate as the spray reagent showed the presence of three spots having similar chromatographic mobilities to those reported by Pazur (77) for nigerose, maltose, and isomaltose (though it seemed unlikely that isomaltose would exist as two peaks on the column.) Chromatography of the material using solvents 1 and 2 using both the descending and the multiple ascent techniques failed to give an adequate resolution of the spots for a quantitative separation using filter paper sheets.

Charcoal - borate chromatography of Fraction C. /

Figure 7

Glucose + Maltose reversion

charcoal-borate column



Charcoal - borate chromatography of Fraction C.

A portion of fraction C (170mg.) was applied to a charcoal-Celite column (60 x 5cm.) previously washed with water and then with borate buffer pH 8.7 (5.72g. sodium borate, 2.47g. boric acid per litre). The column was eluted with 6 litres of the borate buffer with an ethanol gradient 0 - 20% (87). Fractions (50ml.) were collected and their sugar content estimated on a 1ml. aliquot with the phenol - sulphuric acid reagent (55). The results obtained are shown opposite (Figure 7). Fractions were combined as follows:-

C/A	8 - 23
C/B	24 - 27
C/C	28 - 35
C/D	36 - 43
C/E	44 - 59
C/F	60 - 90

The combined fractions were reduced to a small volume under reduced pressure, acidified to pH 3 with dilute sulphuric acid and then extracted with isoamyl alcohol for 24 hours to remove borate ions. (See Section 2). The combined fractions were evaporated to dryness and then re-dissolved in water 5ml. The optical rotation of the solutions were measured and the disaccharide content estimated on an aliquot of the solution after acid hydrolysis.

Fraction C/A 1.2mg., $[\alpha]_D$ + 50° in water, showed the same chromatographic mobility as glucose in solvent 2.

Rotation /

Rotation of glucose +52.6° in water.

Fraction C/B contained no carbohydrate material as shown by acid hydrolysis and cuprimetric estimation of reducing power. Chromatography in solvent 2 using spray reagents 1, 2, 3 and 4 failed to show the presence of a sugar.

Fraction C/C 5.4mg., $[\alpha]_D$ + 186° in water, showed no reaction with Fehlings solution or with aniline oxalate. The sugar also failed to react with triphenyltetrazolium chloride (81) or with 3,5 - dinitro-salicylic acid (82), reactions characteristic of a 1,1-linked disaccharide.

α,α-Trehalose has a rotation of + 175° in water.

Fraction C/D 9.4mg., $[\alpha]_D$ + 136° in water, showed no reaction with Fehlings solution or with aniline oxalate. The sugar failed to react with triphenyltetrazolium chloride but did react with 3,5 -dinitro-salicylic acid; reactions characteristic of a 1,2-linked disaccharide. Kojibiose (2 - O - α- D - glucopyranosyl - D - glucose) has quoted rotations of +133° and +140° (83).

Fraction C/E 33.3mg., $[\alpha]_D$ + 134° in water, reacted with aniline oxalate. On chromatography and on electrophoresis both in borate and in germanate buffer at pH 8.7 showed the same mobility as a sample of nigerose isolated from a partial acid hydrolysate of isolichenin. Acetylation of the material gave a product which after recrystallisation from ethanol /

ethanol had a melting point of 148°C. Quoted values for nigerose $[\alpha]_D$ + 135°; octaacetate, m.p. 147 - 153°C.; synthetic material had a melting point of 151 - 152°C. (76).

Fraction C/F 97.5mg., $[\alpha]_D$ + 125° in water, reacted with aniline oxalate. It showed the same mobility as a sample of maltose on chromatography and on electrophoresis in borate and in germanate buffer pH 8.7. Acetylation gave a product with a melting point after two recrystallisations of 159°C. Quoted values for maltose are $[\alpha]_D$ + 136°; β-octaacetate m.p. 160°C.

Composition of Fraction C. From the results of the borate column chromatography Fraction C was calculated to have the following minimum composition.

Glucose	14.0 mg.
α,α -Trehalose	62.9mg.
Kojibiose	109.5mg.
Nigerose	387.9mg.
Maltose	1136.0mg.

Determination of the ratio of the three disaccharides noted on paper chromatography of the reversion mixture.

Maltose (4g.) and glucose (2g.) were dissolved in sulphuric acid (15ml.; 0.1N.) and heated on a boiling water bath for 5 hours, cooled, neutralised with barium carbonate and the resultant precipitate removed by centrifugation. The precipitate was washed twice with water, the washings and the supernatant combined, and then diluted to 50ml. with distilled /

distilled water in a graduated flask. Aliquots (0.010ml.) were applied to Whatman No. 1 paper and developed with solvent 4 for eight days. The positions of the sugars were revealed by spraying control strips with aniline oxalate reagent. The appropriate areas of the chromatogram were eluted by allowing to stand for twenty minutes in distilled water and then estimating the sugar present on an aliquot (2ml.) using the submicro ferricyanide method of Park and Johnson (84) as follows. Suitable blank determinations were carried out on the chromatography paper.

Reagents.

1) Ferricyanide solution. Potassium ferricyanide (0.5g./litre). Stored in an actinic bottle.

2) Carbonate - cyanide solution. Sodium carbonate (5.3g.) and potassium cyanide (0.65g.) in one litre water.

3) Ferric iron solution. Ferric ammonium sulphate (1.5g.) and Duponol (1g.) in sulphuric acid (1 litre; 0.05N.).

Procedure. The sample (2ml.) was pipetted into an 18mm. stoppered Pyrex test tube to which was added carbonate - cyanide and ferricyanide solutions (1ml. each). After mixing, the tube was heated on a boiling water bath for fifteen minutes, cooled and the ferric iron solution (5ml.) added. The solution was mixed and set aside for fifteen minutes to allow the colour to develop before measuring at 690mμ against a reagent blank in a Unicam SP 600 spectrophotometer.

Results:-/

Results:- using maltose as a calibration standard.

"isomaltose"	~~189.8 µg.~~	13.7 µg.
"maltose"	~~13.7 µg.~~	129.3 µg.
"nigerose"	12.8 µg.	

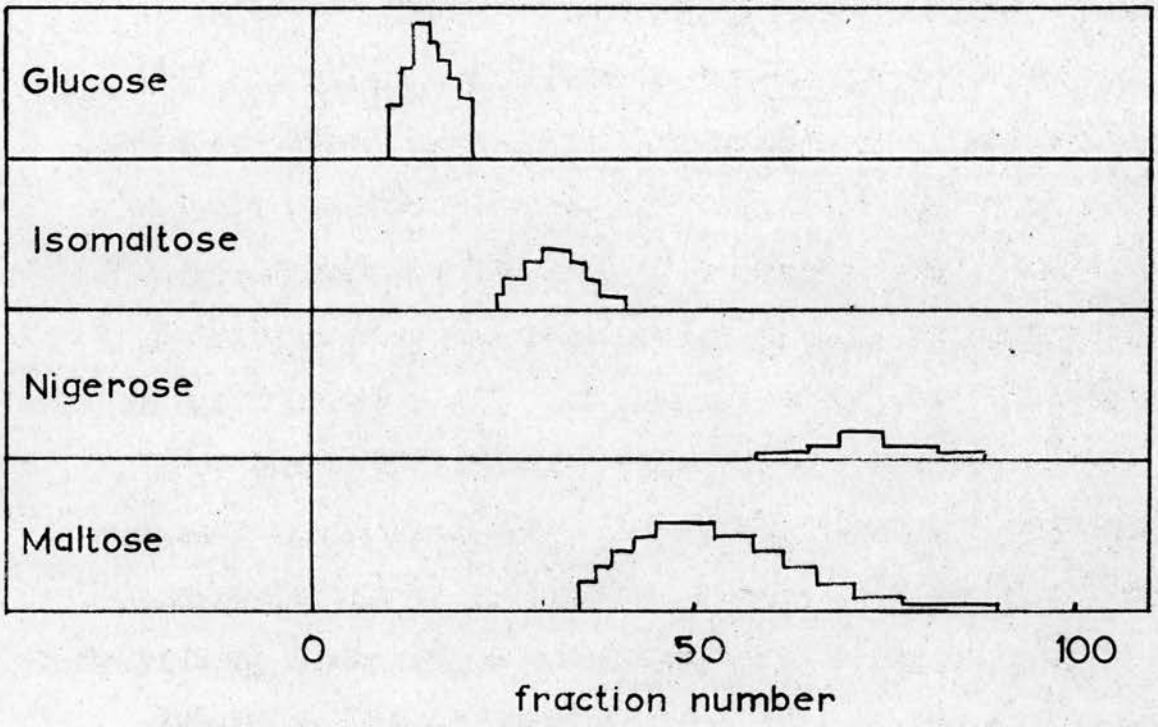
Purity of glucose and maltose used in the reversion.

The glucose and maltose were applied to Whatman No. 3MM paper as a thin syrup and then air dried. After development with solvent 2 and spraying with reagent 1 the glucose appeared to be chromatographically pure. Paper chromatography of the maltose on Whatman No. 3MM paper and identification of the maltose containing band was followed by re-chromatography of the front and back edges of the band on Whatman No. 1 paper in solvent 4 for seven days. This failed to reveal the presence of any other disaccharide components on spraying the paper with reagents 1 and 2. The maltose was thus free from other disaccharides although the presence of a trace of trisaccharide material had been noted earlier on the Whatman No. 3MM paper.

2. Acid reversion of maltose.

Maltose (12.5g.) was dissolved in sulphuric acid (1250ml.; 0.33N.) preheated to 100°C., and then heated on a boiling water bath for 125 minutes. The solution was cooled, neutralised with barium carbonate, centrifuged, the precipitate washed twice with distilled water and the washings and the supernatant solution combined. Paper chromatography /

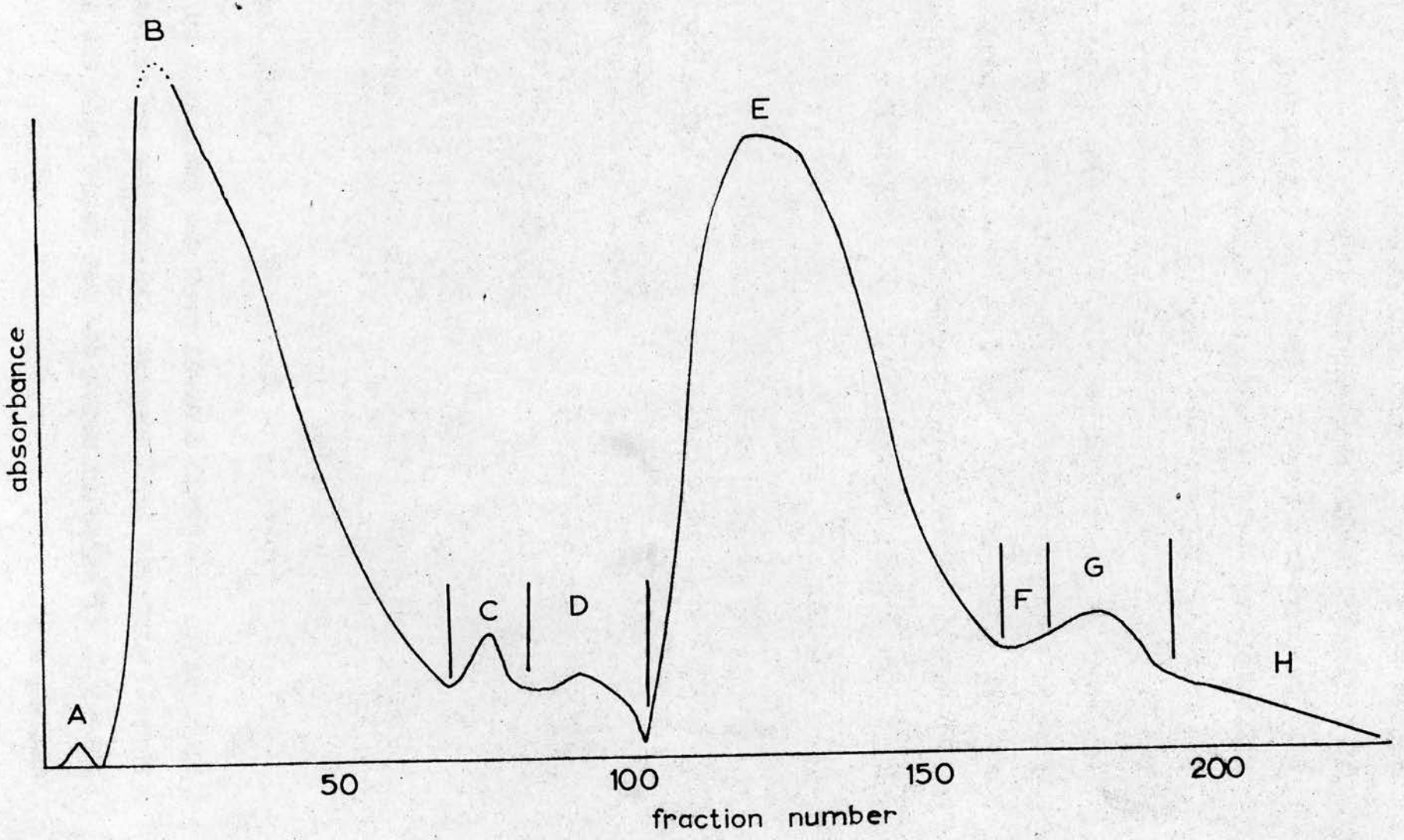
Figure 8 Maltose reversion (1) charcoal column



chromatography of this solution with solvent 2 (two ascents) and spray reagent 1 showed the presence of five spots with the following Rg values 1.0, 0.70, 0.55, 0.37, 0.28 corresponding to the presence of glucose, maltose, isomaltose, panose and an oligosaccharide. The spot with Rg 0.70 (maltose) showed the probable presence of an additional sugar with a slightly higher chromatographic mobility. The solution was reduced in volume to 50ml., applied to a charcoal-Celite column (60 x 7cm.), eluted with water (6 litres) followed by aqueous ethanol (20 litres; 10% $^{v}/v$) and collected in approximately 200ml. fractions, which were reduced in volume to 2ml. The combined aqueous eluate contained no carbohydrate material. Paper chromatography of the ethanolic eluates was carried out with solvent 2 (two ascents) and spray reagent 1. The results obtained are shown opposite (Figure 8). Each fraction was then evaporated to dryness and stored over phosphorous pentoxide under vacuum at 2°C. pending resolution of the components of the glucose and maltose reversion experiments (described on p. 62 et seq.). On completion of this work it was found that the vacuum over the fractions had been lost and that they were now contaminated with a yeast. The reversion was therefore repeated exactly as described above giving a solution which on paper chromatography showed the same five sugars with the same Rg values.

The solution was applied to a charcoal-Celite column /

Figure 9 Maltose reversion charcoal column (ii)



column (45 x 7cm.), eluted with aqueous ethanol (2 litres; 1% v/v) as a linear gradient and finally with 3 litres of 20% v/v aqueous ethanol. Fractions (ca. 50ml.) were collected and their carbohydrate content estimated with the phenol - sulphuric acid reagent on a 1ml. aliquot. The results obtained are shown opposite (Figure 9). Fractions were combined as follows and then evaporated to dryness in tared flasks. Paper chromatography with solvent 2 (two ascents) and spray reagent 1 showed the presence of the following components:-

Fraction	Tube No.	Wt. (g.)	Components
A	4 - 12	---	---
B	13 - 69	8.390	glucose.
C	70 - 82	0.085	glucose.
D	83 - 103	0.048	glucose, laevoglucosan.
E	104 - 163	2.454	glucose (t), maltose, isomaltose, nigerose.
F	164 - 172	0.049	glucose (t), maltose, isomaltose, nigerose, panose (t).
G	173 - 193	0.113	glucose (t), maltose, maltotriose, panose.
H	194 - 230	0.117	glucose (t), maltose (t), maltotriose, maltotetraose (?).

(t) = trace quantity.

Fractions E and F, and G and H were combined.

Fraction A. Although a small peak was indicated by the phenol-sulphuric acid reagent, evaporation to dryness gave only 1mg. of material which did not react with the aniline oxalate /

oxalate, alkaline silver nitrate or periodate - permanganate reagents.

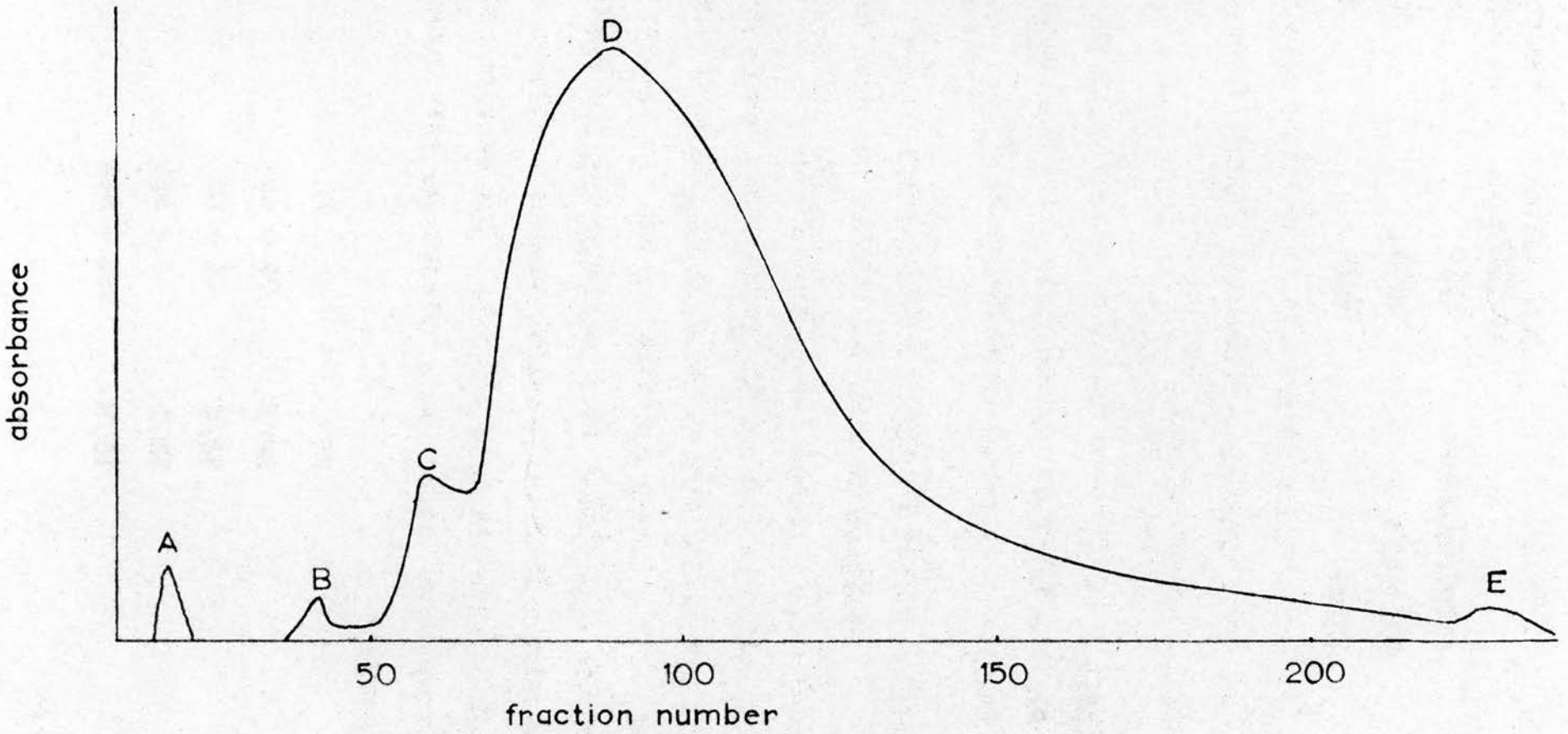
Fraction B. $[\alpha]_D$ + 53° in water, had the same chromatographic mobility of glucose in solvents 1 and 4. Acetylation gave a product which after recrystallisation from ethanol had a melting point of 128°C. Values for glucose are $[\alpha]_D$ + 52.6° and β-octaacetate m.p. 131°C.

Fraction C. Showed the presence of only one component of paper chromatography in solvent 2 using spray reagents 1, 2 and 5 which had the same chromatographic mobility as glucose. The reason for this fraction being a separate peak from fraction B is not known.

Fraction D. Showed the presence of two components on paper chromatography in solvent 2 using spray reagents 1 and 5 with the chromatographic mobilities of glucose and 1,6-anhydroglucose (laevoglucosan) by comparison with authentic samples. The 1, 6-anhydroglucose spot did not react with aniline oxalate and reacted only slowly with alkaline silver nitrate as would be expected for this substance.

The ratio of the two sugars was estimated as follows:-
The fraction was dissolved in water (2ml.) and applied to Whatman No. 1 paper as 0.0025ml. spots. The paper was developed in solvent 1 for 12 hours and dried in a current of warm air. The appropriate sugar containing areas, as indicated by spraying control strips with reagent 1, were cut out as 2" x 2" squares, eluted with water (3ml.) and the sugar content estimated on a 1ml. aliquot using the phenol-sulphuric acid reagent. A paper blank was also carried out.

Figure 10 Maltose reversion charcoal - borate column



	Colorimeter reading.	Concentration* (µg./ml.)
Anhydroglucose	1.10	17.2
Glucose	0.79	12.3
Blank	0.23	3.6

*Based on a calibration using glucose.

Thus Fraction D contained 18.7mg. glucose and 26.2mg. 1,6 -anhydroglucose.

Fractions E and F were combined, dissolved in water (50ml.). Half of this solution was used for paper chromatography; half for charcoal borate chromatography.

Charcoal - borate chromatography of Fraction E F.

Fraction EF solution (25ml.) was applied to a charcoal-Celite column (30 x 5cm.) previously washed with borate buffer pH 8.7 (5.72g. boric acid, 2.47g. sodium borate per litre) and eluted with 10 litres of the borate buffer with a linear gradient 0 - 20% V/v aqueous ethanol. Fractions (ca. 40ml.) were collected and the content of carbohydrate material measured on a 1ml. aliquot using the phenol - sulphuric acid reagent. The results obtained are shown opposite (Figure 10). Fractions were combined as follows:-

EF/A	15 - 22
EF/B	36 - 46
EF/C	47 - 65
EF/D	66 - 220
EF/E	220 - 260

The /

The combined fractions were acidified to pH 3 by the addition of dilute sulphuric acid and the borate ions removed by solvent extraction for 24 hours with isoamyl alcohol.

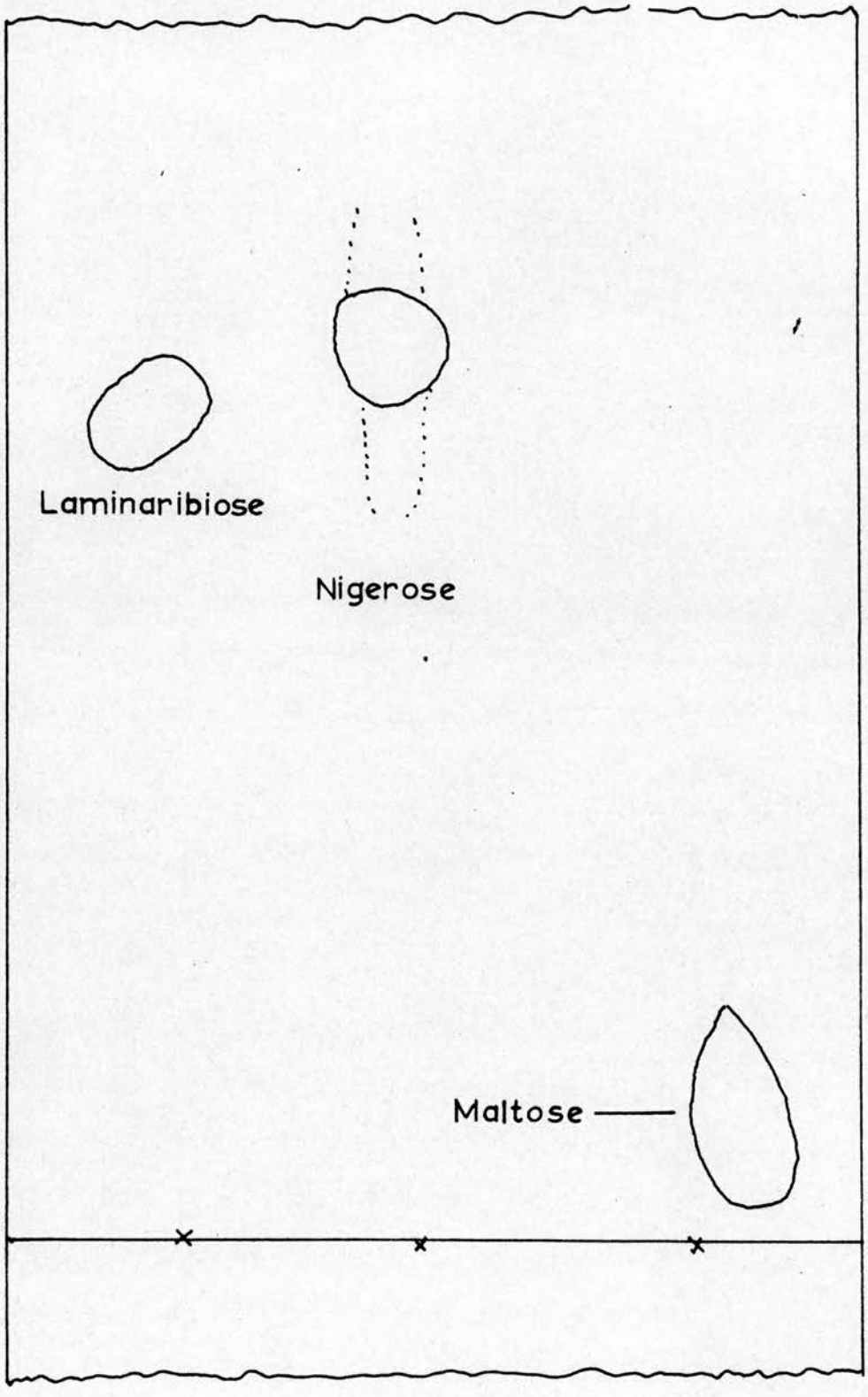
Fraction EF/A was apparently a column artefact in that it did not react with aniline oxalate, alkaline silver nitrate or potassium periodateocuprate when applied to paper.

Fraction EF/B contained a single component, in trace amounts, with the same chromatographic mobility as glucose in solvents 1 and 4.

Fraction EF/C 64.7mg. as estimated by acid hydrolysis, contained a single component with the same chromatographic mobility as isomaltose in solvents 1 and 4. It had $[\alpha]_D$ + 117° in water. Quoted $[\alpha]_D$ for isomaltose 122° .

Fraction EF/D contained two components with the same chromatographic mobilities as maltose and nigerose in solvents 1 and 4. Paper chromatograms tended to streak slightly, probably due to the presence of residual potassium sulphate from the borate extraction, and treatment of the fraction with 3 x 100ml. portions of cold aqueous ethanol (80% $^{v}/_{v}$), to remove the ions, gave a product much reduced in maltose. It was later apparent that some nigerose had been lost at this stage. The product was applied, as a thin syrup, to 6 sheets of Whatman No. 3MM paper and developed with solvent 4 for four days when the nigerose had reached the edge of the sheet /

Figure 11 Electrophoresis of nigerose (EF/C)



sheet with the loss of some of this component. Elution of the nigerose-containing bands with water gave 12.5mg. of a disaccharide as determined by acid hydrolysis which had the same chromatographic mobility as nigerose and an electrophoretic mobility very similar to laminaribiose.(3 - O - α - D - glucopyranosyl - D - glucopyranose). See Figure 11 opposite). Acetylation gave a syrup which eventually crystallised from ethanol. Recrystallisation gave a product melting point 147°C. which on admixture with an authentic sample of nigerose β-octaacetate (kindly supplied by Dr. J.R. Turvey) had a melting point of 147°C.

Fraction EF/E contained a single component in trace amounts with the same chromatographic mobility as an authentic sample of panose (4 - O - α - isomaltopyranosyl - D - glucose) prepared by Dr. W.A.M, Duncan (85).

Preparative paper chromatography of Fraction EF.

A portion of this fraction (650mg. dry wt.) was applied as a thin syrup to four sheets of Whatman No. 3MM paper and developed for four days with solvent 4. The "nigerose" containing bands, located by spraying control strips with reagent 1, were eluted with water (50ml.) and evaporated to dryness giving 9.7mg. disaccharide, as measured by reducing power and acid hydrolysis, with $[\alpha]_D$ + 142° in water. The disaccharide migrated as a single component on paper chromatography, and on electroporesis in borate buffer pH 10.7, having the same mobility as nigerose in the former and similar mobility to laminaribiose in the latter /

latter, (cf. Fraction EF/D). Quoted values for the specific rotation of nigerose are 134 - 139° (76).

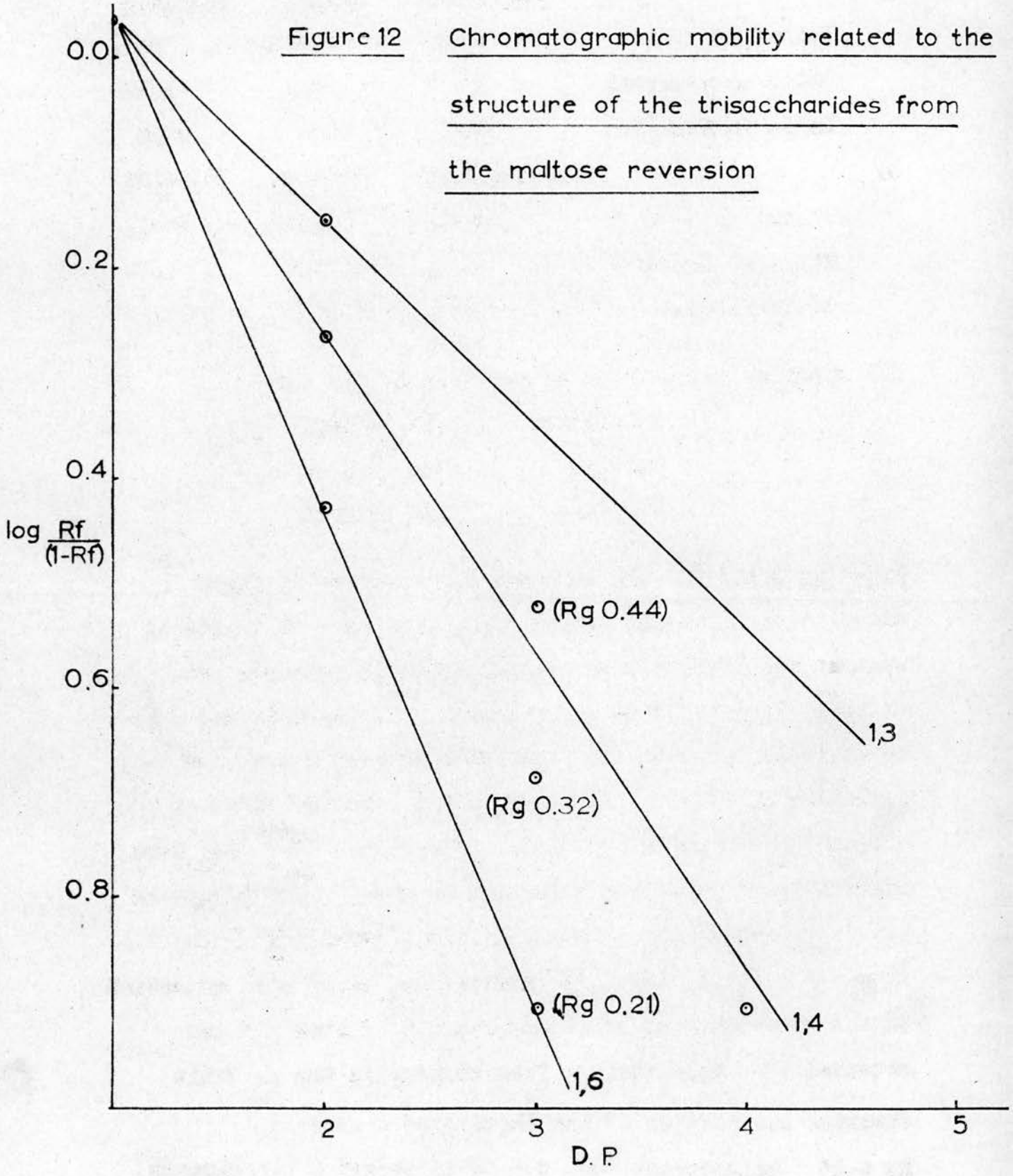
A second portion of this fraction (150mg. dry wt.) was applied to a sheet of Whatman No. 3MM paper and developed for three days with solvent 4. The "maltose" containing band as located by spraying control strips with reagent 1 was eluted with water, evaporated to dryness and then dissolved in 15ml. distilled water. The band was shown to contain a single component with the same chromatographic and electrophoretic mobility as maltose. The component had a specific rotation of + 140° . Quoted values for maltose $[\alpha]_D$ + 136° in water.

Quantitative chromatography of Fraction EF.

The portion of fraction EF not used for preparative chromatography (560mg. dry wt.) was dissolved in water (25ml.) and aliquots (ca. 0.005ml.) were applied as spots to a sheet of Whatman No. 1 paper and developed with solvent 4 for nine days. The positions of the spots were determined by spraying control strips with alkaline silver nitrate. The appropriate sugar containing areas were eluted by immersing in a known volume of water in a petri dish for 20 minutes, with gentle agitation and then estimating the sugar content on an aliquot (1ml.) with the phenol - sulphuric acid reagent (55) using a glucose calibration. Blank determinations were carried out on the paper.

a) /

Figure 12 Chromatographic mobility related to the structure of the trisaccharides from the maltose reversion



a)	Isomaltose	Maltose	Nigerose
Volume	3ml.	30ml.	3ml.
Ratio as measured	1	30	1.19
Ratio in Fraction	77.7	2332	92.6

b)	Isomaltose	Maltose	Nigerose
Volume	10ml.	30ml.	10ml.
Ratio as measured	1	35.9	1.25
Ratio in Fraction	65.6	2355	82.0

The apparent composition of Fraction EF was thus:-

Isomaltose	72	± 6mg.
Maltose	2344	±12mg.
Nigerose	87	± 6mg.

Fraction G and H were combined and examined by paper chromatography using solvent 2 for four days on sheets of Whatman No. 1 paper with control spots of maltose, isomaltose, laminaribiose and glucose. Three spots were revealed on spraying the paper with reagent 1 with the following Rg values 0.44, 0.32, 0.21. The Rf value of glucose in solvent 2 was 0.32. French and Wild (86) have correlated carbohydrate structure to mobility of sugars on paper chromatography. Applying their technique which involves plotting log $\frac{Rf}{1-Rf}$ against degree of polymerisation to the components of fractions G and H Figure 12 was obtained (See opposite). From this graph the possible chemical composition of the three sugars are:-

Rg 0.44 maltotriose, 4 - O - α- nigerosyl - D - glucose.
Rg 0.32 panose, isopanose, (4,6 -di - O - α- glucosyl - D - glucose).
Rg 0.21 isomaltotriose, maltotetraose.

Determination of the ratio of the three disaccharides noted on paper chromatography of the reversion mixture.

Maltose (0.50g.) was dissolved in sulphuric acid (0.33N., 50ml.) which had been preheated to 100°C. and then heated on a boiling water bath for 130 minutes. The solution was rapidly cooled, neutralised with barium carbonate, centrifuged, the precipitate washed twice with distilled water and the washings and the supernatant solution combined. The volume of the solution was adjusted to 10.0ml. with water and aliquots of the solution (0.05ml.) were applied as spots to sheets of Whatman No. 1 paper and developed in solvent 4 for seven days. The papers were dried in a current of hot air and the positions of the disaccharide spots determined by spraying control strips with reagent 1. The appropriate areas of the chromatogram were eluted by allowing to stand in a known volume of distilled water for 20 minutes and then estimating the sugar present on an aliquot (2ml.) using the submicro method of Park and Johnson (84) as described on page 68.

Isomaltose	22.6	g.
Maltose	392	g.
Nigerose	26.4	g.

Purity of the maltose used in this section.

The maltose as applied to Whatman No. 1 paper and developing with solvent 4 for seven days showed the presence of maltose plus a trace of trisaccharide material. The maltose /

maltose was applied to sheets of Whatman No. 3MM paper and developed with solvent 4 for four days. The position of the maltose band was located by spraying marker strips with reagent 1. The front ¼ inch of this band and the ¾ inch in front of that was eluted with distilled water and then evaporated to dryness under reduced pressure. The same procedure was applied to the back ¼ inch of the maltose band and the ¾ inch behind that. Paper chromatography of these portions on Whatman No. 1 paper with solvent 4 for seven days showed the presence of only maltose.

3. β-Amylolysis of Floridean starch.

Floridean starch (0.75g.), isolated by Dr. A.G. Ross (Sample III described by Fleming, Hirst and Manners, (88) , was dissolved in hot water (100ml.) and filtered through a number 4 sintered glass crucible. The solution was transferred to a 500ml. graduated flask to which was added bovine serum albumin (5mg.), β-amylase (6,000 units) and the whole diluted to 500ml. with distilled water and incubated at 37°C.

After 24 hours, when the β-amylolysis limit was 29.6% the digest was poured into an equal volume of ethanol and the resulting opalescent solution reduced to 25ml. by distillation under reduced pressure at 35°C. To this solution was added ethanol (50ml.) with stirring, and the solution centrifuged. The supernatant liquor was evaporated to dryness. Chromatography of the resultant material /

material in solvent 4 for seven days using spray reagents 1 and 2 showed the presence of only maltose.

A portion of the material was applied as a thin syrup to sheets of Whatman No. 3MM paper and developed with solvent 4 for four days. The position of the maltose bands were revealed by spraying control strips with alkaline silver nitrate. The front ¼ inch of the bands and the ¾ inch in front of that were eluted with water, evaporated to dryness and chromatographed with solvent 4 on Whatman No. 1 paper for seven days. Development of the chromatograms with aniline oxalate showed the presence of maltose and under ultra-violet light a very faint fluorescence in front of the maltose spot; development with alkaline silver nitrate showed only maltose while orcinal failed to reveal any ketose containing sugar.

Studies on acid reversion using C^{14}-glucose.

Generally labelled C^{14}-glucose (0.1mc., 0.37mg.) supplied by the Radiochemical Centre, Amersham was used in this work. The material was dissolved in water (0.5ml.), stored in the frozen state and thawed before use. All volumes of radioactive solution mentioned below relate to this solution.

The qualitative evidence obtained below was procured by chromatographing solutions of radioactive material on sheets of Whatman No. 1 paper and placing the air dried chromatogram in direct contact with sheets of Ilford Fast Industrial /

Industrial - G X-ray film for a suitable period of time and then developing the film with Ilford ID - 19 X-ray developer (7 minutes) and fixing with Ilford IF - 22 X-ray acid hardening fixing salt (10 minutes) both solutions being made up as per manufacturers instructions.

Purity of C^{14}-glucose. Paper chromatography of the radioactive solution (0.002ml.) in solvent 1 for 24 hours and subsequent contact with X-ray film for up to 8 days showed that only glucose was present.

Reversion of glucose. To a 5ml. round bottomed flask was added water (0.75ml.), sulphuric acid (2N., 0.15ml.), glucose (10mg.) and radioactive solution (0.1ml.). The stoppered flask was placed on a boiling water bath for 130 minutes, cooled, neutralised with barium carbonate and centrifuged. The precipitate was washed twice with distilled water and the washings and the supernatant liquor combined before evaporating to dryness under reduced pressure. The residual material was dissolved in water (0.1ml.).

A chromatogram was developed in solvent 1 for six hours using 0.006ml. of the solution. Contact with X-ray film for 7 days showed the presence of 1,6 -anhydroglucose, glucose and a region probably containing disaccharides.

Reversion of maltose. To a 5 ml. round bottomed flask was added water (0.75ml.), sulphuric acid (2N., 0.15ml.) maltose (10.0mg.) and radioactive solution (0.1ml.). The stoppered flask was placed on a boiling water bath for 130 minutes, cooled, neutralised with barium carbonate and

centrifuged /

centrifuged. The precipitate was washed twice with distilled water, and the washings and the supernatant solution combined before evaporating to dryness at 35°C. The residual material was dissolved in water (0.1ml.). A chromatogram was developed in solvent 1 using 0.006ml. of the solution. Contact with X-ray film for seven days showed the presence of 1,6 -anhydroglucose, glucose and two di-saccharides identified from chromatographic mobility as nigerose and isomaltose. A chromatogram was developed in solvent 1 for 12 hours with two spots each containing 0.006ml. of the solution flanking a 0.006ml. spot of the glucose reversion solution and left in contact with X-ray film for ten days. Comparison of spot intensities showed that there was much more isomaltose and nigerose in the maltose reversion.

Partial acid hydrolysis of amylopectin (potato var. Gt.Scot.) using ~~of~~ C^{14} glucose.

Amylopectin (9mg.) was dissolved in water (0.65ml.) with heating. To the cooled solution was added radioactive glucose solution (0.2ml.) and sulphuric acid (2N.; 0.15ml.) and the flask placed on a boiling water bath for two hours. The solution was cooled, neutralised with barium carbonate, centrifuged, the precipitate washed with distilled water and the washings and the supernatant combined before evaporating to dryness under reduced pressure. The residual material was dissolved in water (0.1ml.). An aliquot (0.006ml.) was applied /

applied to Whatman No. 1 paper and developed with solvent 1 for twelve hours before placing in contact with X-ray paper for five days. Development of the film showed the presence of glucose, isomaltose, nigerose and a large though very faint spot of maltose. Comparison with the film containing the glucose and maltose reversions showed that the partial hydrolysate was similar to the maltose reversion but not the glucose reversion in the intensities of the disaccharide spots.

DISCUSSION.

1. Acid reversion of a mixture of glucose and maltose.

The reversion was carried out under the conditions of Pazur and Budovitch (77) with the exception that 0.1N sulphuric acid was used in preference to 0.1N hydrochloric acid since neutralisation with barium carbonate and centrifugation gives a solution largely free from ions and at a neutral pH, in comparison with the highly ionic solution and slightly alkaline pH obtained by neutralising the hydrochloric acid with sodium carbonate. No attempt was made to remove the maltose and glucose using baker's yeast. Paper chromatography, as used by these authors, was found to be unsuitable in that maltose and nigerose could not be completely separated. In the present work, charcoal column and charcoal-borate column chromatography has been preferred.

The glucose used in the reversion was chromatographically pure whilst the maltose though free from other disaccharides contained a minute trace of trisaccharide material.

Pazur and Budovitch tentatively identified glucose, maltose, nigerose, isomaltose, and cellobiose in the reversion mixture by their paper chromatographic mobility. In this work paper chromatography has shown the presence of five sugars which by comparison with controls have been shown to be glucose, maltose, nigerose, isomaltose and a trisaccharide.

The following sugars were isolated from the reversion /

reversion mixture (Table 7). The yields were calculated from aliquots of the reversion mixture.

Table 7.

Reversion products from a mixture of maltose and glucose.

Glucose	26.429g.
Maltose	3.971g.
Isomaltose	0.195g.
Nigerose	0.388g.
α,α-Trehalose	0.063g.
Kojibiose	0.110g.

Repetition of the reversion on a smaller scale and analysis of the three major disaccharide spots separated by paper chromatography by the Park and Johnson ferricyanide method (84) gave the following results adjusted to the scale of the original reversion.

Maltose	3.880g.
Isomaltose	0.410g.
Nigerose	0.384g.

α,α-Trehalose will not be estimated by this method and it is doubtful if Kojibiose, which does not reduce the Somogyi reagent will be estimated. The higher yield of isomaltose in the small scale experiment was expected in view of the loss of some of this component from the large scale experiment during one attempt to free it from traces of maltose and glucose.

This /

This work has confirmed the yield of nigerose obtained by Pazur and Budovitch, and has also shown that the material identified by them as nigerose has a specific rotation of +134° in water in agreement with the now accepted values for nigerose and in contrast to their apparently anomalous value of +87° .

An interesting feature of the reversion experiments is the apparent absence of β-linked sugars in contrast to the results obtained from the reversion of glucose alone (66).

2. Reversion of maltose.

The maltose used in this work was shown to be free from other disaccharides but contained a trace of apparent trisaccharide material.

The reversion conditions employed were identical to these used by Peat and his co-workers (66) and by Peat, Turvey and Evans for the hydrolysis of Floridean starch (71). In view of the successful application of charcoal-borate chromatography to the resolution of the components of the glucose and maltose reversion this method was preferred to that of Peat and his co-workers who used paper chromatography and electrophoresis.

The reversion has been repeated three times and in each case the same five sugars have been shown to be present on paper chromatograms. The relative amounts of the various sugars appeared to be identical.

The following sugars were isolated from the reversion mixture (Table 8). The composition was

calculated /

calculated from analysis of aliquots.

Table 8.

Reversion products from maltose.

Laevoglucosan	26mg.
Glucose	8494mg.
Maltose	2344 ± 12mg.
Isomaltose	72 ± 6mg.
Nigerose	87 ± 6mg.

The separation of maltose and nigerose on charcoal-borate chromatography presented unexpected difficulties and chromatography on thick paper had to be used. In an attempt to obtain the resolution of these two sugars, for quantitative estimation and characterisation, a considerable portion of the nigerose was lost. A total of 21.2mg. of the nigerose was however isolated and characterised representing 31.2mg. of the whole sugar in the reversion mixture. A small scale reversion experiment and estimation of the three disaccharides separated on paper chromatography, by the method of Park and Johnson (84), gave the following results adjusted to the scale of the original reversion.

Maltose	1960mg.
Isomaltose	113mg.
Nigerose	132mg.

These results are of the same order of magnitude as those obtained in the original reversion experiment. They provide both an independent proof of the much higher yield /

yield of nigerose to be expected from a maltose reversion in comparison with the reversion of glucose and also confirms the observation that the yield of isomaltose is slightly less than that of nigerose.

In common with the results obtained from the acid reversion of a mixture of glucose and maltose, and in contrast to the results obtained from the reversion of glucose alone under the same conditions (66), no β-linked disaccharides have been isolated.

Vernon and his co-workers (89), in a study of the acid catalysed solvolysis of glycosides in methanol, have shown that the dependence of rate with acidity and with structure is best explained by a carbonium ion mechanism. These workers have shown that the reaction involves the inversion of the configuration of C_1 in direct contrast to the results obtained above. While direct comparison between the reaction conditions carried out by Vernon and his coworkers and those used in this work is not possible the difference in the results is nevertheless surprising.

In this reversion no α,α-trehalose or kojibiose was isolated though it would seem unlikely that these sugars were absent. The occurrence of these two sugars may possibly account for the apparent discrepancy in the amounts of isomaltose isolated from the two halves of fraction EF. It is feasible that the α,α-trehalose and kojibiose peaks would be masked by the isomaltose peak on charcoal borate chromatography and the apparent amount of isomaltose isolated by this method would be greater than that obtained by paper chromatography.

3. β-Amylolysis of Floridean starch.

The β-amylolysis of a sample of Floridean starch has been carried out under the identical conditions of polysaccharide and enzyme concentration as used by Peat, Turvey and Evans (71). No buffer was used in the enzyme digest which was prepared using distilled water. Ethanol was added to the digest in order to deactivate the enzyme, precipitate the β-limit dextrin and to obtain the liberated maltose in solution free from ionic material and from the dextrin. This method was preferred to that of the above authors which involved heating the enzyme digest containing buffer at 100°C. to inactivate the enzyme and dialysis in the presence of buffer apparently at room temperature for 36 hours. The β-amylolysis limit of the polysaccharide after 24 hours was 30% which is lower than that obtained by Peat, Turvey and Evans for their sample of Floridean starch but is comparable with the value of approximately 37% obtained by Fleming and co-workers (88), for this sample.

Paper chromatography of the maltose failed to show the presence of any other disaccharide material while chromatography of the leading edge of the maltose band from a sheet of Whatman No. 3MM paper showed the presence of a minute trace of an additional component with a chromatographic mobility corresponding to nigerose or maltulose. The amount of this component was so small as to be barely visible under ultra-violet light on a chromatogram sprayed with aniline /

aniline oxalate and was not visible using either the orcinol or the alkaline silver nitrate reagents. In view of the difficulty in detecting this component it is very doubtful if any structural significance can be attached to this finding.

4. The use of C^{14}-glucose in reversion studies.

The results obtained from the use of C^{14}-glucose in this work are only qualitative in that a visual comparison of autoradiograms produced under controlled conditions can be made.

Comparison of reversions of glucose and of maltose containing C^{14}-glucose under the conditions of Peat and co-workers (66) has confirmed the quantitative work described earlier in that much higher quantities of isomaltose and nigerose are formed from reversion with maltose than with glucose. It has not been possible to obtain any comparison of the intensities of the maltose spots in view of the dilution of the radioactive maltose formed by reversion with unhydrolysed maltose in the reversion using that sugar.

Comparison of the radioactive disaccharides formed by reversion during the hydrolysis of a sample of potato amylopectin with those of the glucose and maltose reversions has shown that there is a much closer similarity between the amylopectin hydrolysate and the maltose reversion than there is between the amylopectin hydrolysate and the glucose reversion. The presence of a radioactive fraction, apparently trisaccharide, in both the amylopectin hydrolysate and /

and the maltose reversion has also been noted.

The use of C^{14}-glucose and C^{14}-maltose, with a higher level of radioactivity than has been used in this instance, in conjunction with the chromatographic separation procedure of Ough (91) and the use of counting techniques for the measurement of the amounts of the radioactive disaccharides could result in a rapid method for the study of the acid reversion of sugars.

The conclusions which can be derived from this work are as follows:-

1) Glucose is not a suitable control for acid reversion studies on the hydrolysis of glucose-containing polymers. It would appear, in agreement with a suggestion by Peat, Whelan, Edwards and Owen (66), that for a polymer containing largely α-1,4-linkages, the disaccharide containing that linkage (maltose) must be used.

2) The use of radioactive sugars in conjunction with an adequate method of paper chromatography may provide a much more rapid method of study of the acid reversion of sugars than has been developed hitherto.

3) The detection of a <u>trace</u> amount of a sugar other than maltose from the β-amylolysis of Floridean starch is of doubtful significance.

4) Repetition of the work of Pazur and Budovitch (77) has confirmed (a) their yield of nigerose and (b) that the rotation of $+87^{\circ}$ obtained by these workers for this disaccharide was anomalous.

5) The absence of β-linked disaccharides in both the reversions /

reversions carried out may indicate that reversion occurs largely by attack of the non-reducing glucose unit of maltose as it is hydrolysed.

6) The occurrence of nigerose in partial acid hydrolysates of Floridean starch, amylopectin and glycogen is probably not an indication that α-1,3-linkages occur in these polymers. The development of this conclusion for each of the three polysaccharides is as follows:-

a) Floridean starch. The yield of nigerose (ca. 87mg.) isolated in the present experiments may represent a maximum value under the experimental conditions, in that acid hydrolysis of the maltose and acid catalysed transglucosylation are occurring throughout the experimental period (125 minutes) whereas in the partial acid hydrolysis of starch, depolymerisation of polysaccharide material is the major reaction and transglucosylation may be limited. This yield, however, as indicated by the hydrolysis of amylopectin in the presence of C^{14}-glucose, is suitable for comparison with the yields of nigerose isolated from the partial acid hydrolysis of starch-type polysaccharides.

Peat and his co-workers (71) isolated 34mg. of nigerose from 12.5g. of Floridean starch under the same conditions as employed in this work, and it now seems probable that all of this material could be accounted for by acid catalysed transglucosylation during hydrolysis. The isolation of trisaccharides containing 1,3-linkages from the partial acid hydrolysate may not now be significant

in /

in view of the isolation of a trisaccharide fraction (130: 4mg.) from the acid reversion of maltose which, from chromatographic evidence, may contain a 1,3-linked trisaccharide. The liberation of nigerose on β-amylolysis of the starch (90) has not been confirmed.

It would thus seem that the presence of 1,3-linkages in Floridean starch has not been rigidly established and further methods of structural analysis are required before an unambiguous answer can be given to this problem.

b) Amylopectin. Wolfrom and Thompson (69) isolated 350mg. nigerose β-octaacetate (equivalent to 170mg. nigerose) from 130g. waxy maize starch (equivalent to 144g. glucose) on hydrolysis with 0.1N hydrochloric acid at 0.4% concentration. These results indicate the production of ca. 125mg. nigerose per 100g. glucose. From the maltose reversion experiments described in this work the yield of nigerose per 100g. maltose (expressed as glucose) is 660mg. While no direct comparison between these results is possible in view of the different acid and sugar concentrations and period of heating, it is feasible that the amount of nigerose obtained by Wolfrom et al is of the same order of magnitude as that expected from acid catalysed transglucosylation.

c) Glycogen. Wolfrom and Thompson (72) isolated 2mg. nigerose β-octaacetate from 96g. beef liver glycogen. In view of the amount of nigerose isolated from the acid reversion of maltose it would seem inconceivable that the 2mg. of octaacetate, equivalent to only 1mg. of disaccharide has any structural significance.

Since /

Since the supporting evidence for 1,3-linkages in amylopectin and glycogen based on periodate oxidation has now been shown to be inconclusive, the present results suggest that both amylopectin and glycogen do not contain α-1,3-linkages.

SECTION 4.

The occurrence of fructose in starch type polysaccharides.

INTRODUCTION.

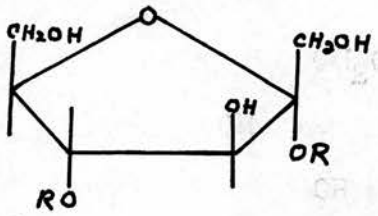
There are three reports of the isolation of maltulose (4 - O - α-D - glucopyranosyl - D - fructose) from α- amylolytic digests of starch type polysaccharides. Peat, Roberts and Whelan (92) isolated 4.7% of maltulose from the glycogen from the livers of pregnant does. Maltulose has been isolated from waxy maize starch by Radomski and Smith (93) in a yield of 0.98% and 1.76% confirming the qualitative evidence of Whelan and Roberts (94). Peat, Roberts and Whelan (94) also noted that fructose was detected in the maltotriose fraction and in the α-limit dextrin fraction arising from the degradation of their glycogen. In each case suitable control experiments showed that this sugar was not an artefact of the procedure used, and the conclusion which must be drawn from this work is that D - fructose occurs as a minor constituent of amylopectin and glycogen.

Although it is known that fructose may occur to a small extent in dextran (95), the possibility still exists that the fructose arises from small amounts of a fructosan impurity known to occur in conjunction with the dextran. There do not appear to be any other reports of small amounts of fructose in glucosans. On a biochemical basis this is

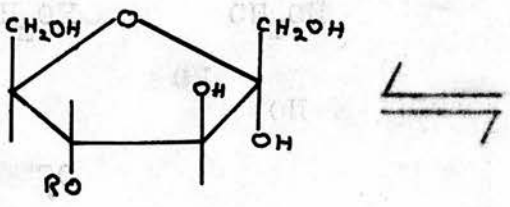
not /

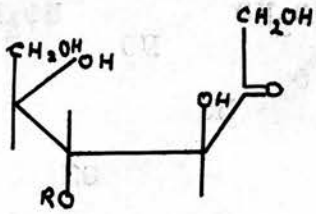
not surprising in view of the necessity for a different enzyme system for the introduction and removal of an anomalous sugar unit into an otherwise homogeneous molecule. It is thus unlikely though by no means impossible, that fructose should occur as a constituent of amylopectin or glycogen.

The fructose containing unit of maltulose, as an integral part of the structure of amylopectin (I),

I. 

would not appear to be readily susceptible to periodate attack, assuming that the fructose unit is glycosidically linked at C_2 as in the known fructosans. Under the conditions of periodate oxidation the formic acid released may effect hydrolysis at C_2 of the fructose unit giving structure II below. This compound will exist to a slight extent as its acyclic form (III) which is susceptible to periodate attack.

II. 



III.

It is thus not surprising that no evidence has been presented to date of the isolation of fructose subsequent to the periodate oxidation of starch type polysaccharides.

The /

The aim of the present work has been to carry out an α-amylolysis of waxy maize starch at an enzyme concentration such that the minimum conversion of maltotriose to maltose and glucose was effected and to examine the maltose and maltotriose for the presence of fructose.

The experimental work was carried out in collaboration with Mr. J.K. Smith who was responsible for the preparation of the enzyme and the enzyme digest.

EXPERIMENTAL.

Preparation of a salivary α-amylase (Mr. J.K. Smith).

The α-amylase was prepared from human saliva (1700ml.) as described by Bernfeld (96) giving 910mg. of a freeze dried powder with an activity of 0.18 units/mg. as determined by the method of Fischer and Stein (97) as modified by Manners and Wright (98). The preparation was not entirely free from maltase activity in that one unit of enzyme acting on maltose (20mg.) for 24 hours at pH 7 and at 35°C. converted 2.5% of the maltose into glucose.

Determination of the enzyme concentration required to give an apparent conversion of waxy maize starch into maltose of 60%.

Digests were set up as follows:- to a 25ml. graduated flask was added starch solution (2ml.; 9.08mg./ml.), phosphate citrate buffer (5ml.; 0.2M.; pH 7.0) and sodium chloride solution (1ml.; 0.1% W/v). A weighed amount of the enzyme preparation was added followed by water to a final volume of 25ml. The digests were covered with toluene and incubated at 35°C.

Units enzyme per digest	Time of incubation (hours)	Apparent conversion to maltose (%)
10	19	80
	67	91
5	19	78
	67	84

Units /

Units enzyme per digest	Time of incubation (hours)	Apparent conversion to maltose (%)
1	19	72
	67	76
0.1	43	54
	67	64
0.01	43	20
	67	25

From these results it appeared that 0.1 units of enzyme/20mg. polysaccharide would give the desired apparent conversion to maltose of approximately 60%.

α-amylolysis of waxy maize starch IV (Mr. J.K. Smith).

Starch (20g.) was dissolved in sodium hydroxide solution (2 litres; 2N.) with the aid of heating, filtered through a glass wool pad, and neutralised with sulphuric acid (4N.). Phosphate-citrate buffer (0.2M; pH 7.0; 150ml.) was then added followed by sodium chloride (200mg.). To this solution was added the enzyme preparation (600mg.) followed by distilled water to a final volume of four litres. The carbohydrate content of the solution was determined on an aliquot (5ml.) by acid hydrolysis and cuprimetric estimation of the liberated glucose. The solution was covered with toluene and incubated at 35°C. After 66 and 90 hours aliquots (5ml.) were removed, diluted to 100ml. and the apparent conversion to maltose determined cuprimetrically on an aliquot (5ml.). The values at both 66 and 90 hours were identical at 59.9%. The digest was stored overnight at /

at 2 - 4°C., centrifuged and the supernatant solution heated to 80°C. for five minutes to inactivate the enzyme, cooled, and evaporated to dryness under reduced pressure at 40°C.

Fractionation of the products of the α-amylolysis of waxy maize starch IV.

The dried material was extracted six times with 200ml. portions of hot aqueous ethanol (80%^{v}/v) which were combined and reduced to a final volume of 100ml. under reduced pressure. This solution was applied to an Ultrasorb charcoal/Celite column and eluted as follows:-

Ethanol concentration (% v/v)	Fraction number	Eluate volume (litres)
0	-	2.8
2	-	5.1
5	-	5.0
10	1	3.5
	2	3.8
	3	4.4
15	1	4.2
	2	1.2
20	-	7.6
30	-	5.0

Fractions were evaporated under reduced pressure to a final volume of 10ml. and frozen until required for use.

Chromatography of column fractions.

Fractions were thawed at room temperature and applied to a sheet of Whatman No. 3MM paper which was developed /

developed in solvent 4 for four days. Development with the orcinol spray reagent failed to reveal any ketose containing sugars. Development with reagent 1 gave the following results:-

Ethanol (%, v/v)	Fraction	Glucose	Maltose	Maltotriose
0	-	-	-	-
2	-	-	-	-
5	-	++	-	-
10	1	+	-	-
	2	+	-	-
	3	t	++	-
15	1	-	+++	-
	2	-	++	-
20	-	-	t	+++
30	-	-	-	++

The fractions containing glucose, maltose, and maltotriose were each combined, evaporated to dryness in tared flasks and stored under vacuum over phosphorous pentoxide before weighing.

Glucose	0.64g.
Maltose	4.34g.
Maltotriose	2.63g.

Chromatography of these fractions in solvent 4 for three days on Whatman No. 3 MM paper, as expected, failed to show the presence of any fructose containing spots with the orcinol reagent.

Compound chromatography of the glucose, maltose and maltotriose fractions.

A portion of each of the fractions was applied as a thin syrup to sheets of Whatman No. 3 MM paper and developed with solvent 4 as follows - the glucose containing sheet for two days, the maltose for four days and the maltotriose for six days. The sugar containing bands were located by spraying control strips with reagent 1 and the front $\frac{1}{4}$ inch of each of the bands together with the $\frac{3}{4}$ inch in front of that was eluted with distilled water and evaporated to dryness under reduced pressure at 35°C. Portions of these leading edge fractions were applied as spots to sheets of Whatman No. 1 paper and developed with solvent 4 for two, four, and six days as described above. Spraying with the orcinol reagent showed that both the maltose and maltotriose fractions contained ketose sugars in that they showed barely visible green spots in daylight more readily seen under ultra violet light. Subsequent overspraying with reagent 2 showed that these spots did not coincide with maltose or maltotriose containing areas.

Chromatography of the maltose leading edge fraction in solvent 4 for five days on Whatman No. 1 paper showed that the ketose component had the same chromatographic mobility as an authentic sample of maltulose.

A portion of the maltose leading edge fraction was applied to a sheet of Whatman No. 1 paper, developed in solvent 4 for five days, the position of the ketose containing /

containing band determined by spraying marker strips with the orcinol reagent and the material eluted from this band. Electrophoresis at 750 volts for five hours and subsequent spraying with the orcinol reagent showed that the ketose material had the same electrophoretic mobility as an authentic sample of maltulose.

DISCUSSION.

The α-amylolysis of a sample of waxy maize starch has qualitatively confirmed the results obtained by Peat, Whelan and Roberts (94) and by Radomski and Smith (93) in that a ketose containing disaccharide, apparently maltulose, has been detected. This work has also confirmed the observation (92) that ketose material is contained in the maltotriose fraction from the α-amylolysis digest.

"Normal" paper chromatography of the components of the digest failed to reveal the presence of the ketose containing sugars. Only after "compound" chromatography, involving the elution and concentration of the possible ketose containing areas of the chromatograms, and rechromatography were the sugars detected with the orcinol reagent. The ketose spots obtained were barely visible in daylight, though the use of ultraviolet light enabled them to be viewed comparatively easily. In view of the difficulty in detecting the ketose containing sugars it would seem that the amounts present may not exceed 1% of each of the fractions.

The logical conclusion from this preliminary study is that fructose is a small but integral part of the structure of amylopectin.

As pointed out previously, the release of formic acid /

acid on periodate oxidation may hydrolyse the glycosidic linkage at C_2 on the fructose unit leaving this unit susceptible to oxidation. Periodate oxidation under buffered conditions, possibly at pH 5, may prevent this hydrolysis. Subsequent destruction of the excess periodate, reduction of the polymer polyaldehyde, and partial acid hydrolysis with 0.1N. sulphuric acid should release free fructose if any is present. Any under-oxidation giving residual glucose should not interfere since the unoxidised glucose units would occur as glucosyl erythritol and the separation of this compound from fructose should present no difficulties by paper chromatography.

A second approach to this problem would involve measuring the viscosity of amylopectin in 0.1N acid solution over a period of three to four hours at or near room temperature. The conditions suggested are those usually employed for the total hydrolysis of fructosans (99). The results obtained from the α-amylolytic studies would seem to indicate a random distribution of the fructose units and hydrolysis of these units should cause a reduction in viscosity of the amylopectin sample if indeed those units are present.

SECTION 5.

The structure of the starch-type polysaccharide formed by Polytoma uvella.

INTRODUCTION.

The production of starch-type polysaccharides is a characteristic feature of the higher land plants, but it is also found in the lower orders of the plant kingdom including some unicellular organisms. The structures of the polysaccharides from such organisms are of interest, not only from a chemical stand point, but also in that they may have some taxonomic value.

Fogg (100) states that the Chlorophyceae tend to be ancestral to land flora having starch and fat as reserve materials. This statement is supported by the isolation and characterisation of starch from Polytomella coeca (101), Dunaliella bioculata (102), Chilomonas paramoecium (103), and Nitella translucens (104). Chladophora rupestris, also a member of the Chlorophyceae, was apparently an exception to the statement in that a laminarin-type polysaccharide was the main reserve carbohydrate (105). McKinnell and Percival (106) have however isolated small quantities of an iodophilic glucan from this alga with starch-like properties.

Polytoma obtusum, a member of the Chlorophyceae was shown (107) to contain a glucosan which stained blue with iodine and which was degraded by amylases from several sources, releasing an unidentified substance which reacted with /

with Fehlings solution. Another member of the same genus, Polytoma uvella, was shown by Pringsheim (108) to contain intracellular iodophilic granules which were attacked by amylases giving sugars which reduced Fehlings solution.

The aim of the present work has been to isolate the starch-type polysaccharide of the flagellated protozoan Polytoma uvella in a pure form, to examine its structure and if possible to study the properties of its component fractions.

EXPERIMENTAL.

Depigmented cells of Polytoma uvella were kindly supplied by Dr. J.F. Riley. These cells (Polytoma uvella strain (62-2A) obtained from the Culture Collection of Algae and Protozoa, Cambridge, April 1960) were the product of 14 batches. Each batch, utilised 36 x 2 litre flasks containing 400ml. of medium, was incubated at 24°C. and harvested after 6 days. The culture medium was prepared as follows:-

Sodium acetate 0.2%; "Oxoid" yeast extract 0.1%; "Oxoid" peptone 0.1%. The cells from each batch were stood in approximately 100ml. methanol for 1 - 3 days, the coloured solution was decanted off and the cells were washed with methanol until colourless.

The cells were disrupted in an M.S.E. ultasonic disintegrator (18,000 - 20,000 cycles/sec., power output 60 watts.). A methanolic suspension of the cells (approximately one quarter of the total material in 25ml. methanol) was treated in the disintegrator for six minutes and the resultant material centrifuged at 4,000 r.p.m. for 20 minutes. The supernatant liquid was discarded whilst the residue was washed several times with distilled water to remove the remaining methanol. The material from the cells was then deproteinised by the method of Clark and Stone (109). The cellular material was digested with a 1% trypsin solution buffered at pH 7.6 with 0.1M phosphate buffer for 24 hours at 37°C. The residue from the digest

was /

was extracted three times at 0°C. with 90% saturated aqueous urea solution. No attempt was made to further deproteinise the residue by Sevag's (110) method, since a small scale experiment had shown that a very large proportion of the granular material was held at the interface between the organic and aqueous phases, and, that this material could not be removed by centrifugation at 5,000 r.p.m. for 30 minutes. The residue from the urea extractions was washed once with distilled water followed by 50% ethanol (twice), methanol and then ether giving a pure white powder, weight 3.0g. Microscopic examination of the powder did not reveal any whole cells or cellular debris but showed oval weakly birefringent granules.

Total acid hydrolysis of the starch.

Approximately 10mg. starch was heated in 10ml. 3N. sulphuric acid for 2.5 hours. The solution was neutralised with barium carbonate, centrifuged and the supernatant liquid evaporated to dryness under vacuum. A chromatogram of the resulting material on Whatman No. 1 paper using solvent 2 overnight showed only one spot when sprayed with aniline oxalate which corresponded exactly with a glucose control spot. Glucose is thus the only reducing sugar present in the starch.

Percentage Purity:- 14.2mg. starch previously dried under vacuum over phosphorous pentoxide was dissolved in 1ml. 1N potassium hydroxide neutralised with dilute hydrochloric acid and the volume adjusted to 20ml. in a standard flask.

1ml. /

1ml. aliquots were removed and subjected to acid hydrolysis using 2N sulphuric acid for three hours. The glucose released was estimated using the Shaffer Hartman reagent and from this the polysaccharide content of the sample was calculated.

Apparent polysaccharide content = 13.6mg.
∴ Purity = 96.0%

Blue value:- Using the same polysaccharide solution as used for the estimation of percentage purity 5mg. of the polysaccharide (7.40ml.) was pipetted into a 500ml. standard flask, 5ml. iodine solution (0.2% iodine, 2% potassium iodide) and 0.25ml. 6N hydrochloric acid was added. The solution was diluted to 500ml. with distilled water and the absorbance of the solution measured between 450mμ and 750mμ in a Unicam S.P. 500 spectrophometer using 4cm. cells. The Blue value (B.V.) is taken as the absorbance at 680mμ.

Polytoma Starch B.V. = 0.244

Amylose content:- A potentiometric iodine titration was carried out on 36.4mg. of the starch as described in the General Methods section. The iodine affinity of the starch measured as g. iodine bound per 100g. polysaccharide (% iodine bound) was 3.1%. The iodine affinity of a pure amylose is approximately 19.5%. Applying this figure to the Polytoma starch the percentage amylose present was 15.9.

Attempted fractionation of the starch with n-butanol.

518Mg. starch was dispersed in 50ml. boiling water

under /

under an atmosphere of nitrogen for 1 hour. The solution was filtered hot through a sintered glass crucible (porosity No. 2) giving 26.3mg. hot water insoluble material. The opalescent solution was boiled with 20ml. butanol under nitrogen for 10 minutes and then cooled overnight. The butanol complex was centrifuged and the supernatant liquid treated with 4 volumes ethanol. The precipitate from the supernatant liquid was collected by centrifugation and further treated by the addition of methanol followed by ether and dried over phosphorus pentoxide under vacuum giving 14.8mg. "amylopectin". The butanol complex was dissolved in boiling water (50ml.) under nitrogen, re-precipitated by the addition of butanol as above and centrifuged. The precipitate was triturated with ethanol followed by methanol and then ether and finally dried under vacuum over phosphorus pentoxide. Yield 440mg. "amylose".

Potentiometric iodine titrations of butanol fractions.

36.4mg. "amylose" and the whole of the "amylopectin" and "insoluble" fractions were used in these estimations. Assuming an iodine binding power for amylose of 19.5% the percentage amylose found in each of the fractions was as follows:- "amylose" 15.6%. "amylopectin" 12.1%, "insoluble" 11.8%.

Attempted fractionation of the starch with thymol.

Starch (568mg.) was dissolved by heating in boiling water (100ml.) under nitrogen for two hours. The solution was /

was allowed to cool to 80°C. and approximately 1g. powdered thymol was added. The solution was allowed to cool to room temperature with continuous stirring and then set aside for three days to allow the complex to "age". The solution was centrifuged in an M.S.E. refrigerated centrifuge set at -5°C. with a high speed attachment at 17,000 r.p.m. for 30 minutes. The supernatant solution was decanted off carefully and then freeze dried. Yield 7.0mg. The precipitate was triturated with 25ml. portions of ethanol, methanol, and finally ether, before being dried under vacuum over phosphorus pentoxide at room temperature for several days. Yield of "Amylose" 287mg.

Thus no fractionation had been effected.

Liquid ammonia pretreatment of the starch and attempted fractionation with thymol.

Starch (ca. 200mg.) was placed in a hard glass test-tube (4 x 0.5ins.) and covered with liquid ammonia (10ml.). The lower half of the test-tube was inserted into a "Drikold" - acetone slurry to reduce the amount of ammonia boiling off and then set aside for one hour. At the end of this period the tube was placed in a current of warm air and the ammonia allowed to evaporate over a period of three hours. The resultant semi-solid material dissolved readily in water (5ml.) to give a clear solution which was added to boiling water (200ml.) under an atmosphere of nitrogen. The solution was boiled for twenty minutes with continuous stirring, allowed to cool to 80°C.

and /

and then powdered thymol (0.5g.) was added. The solution was allowed to cool slowly with stirring and then set aside for 45 hours. The solution was centrifuged. The precipitate was washed with ethanol, followed by ether and placed under vacuum over phosphorus pentoxide for 24 hours. Yield 215mg. The supernatant solution was evaporated to ca. 20ml. under reduced pressure and ethanol (80ml.) added. The solution was centrifuged; the precipitate was washed with ethanol followed by ether and dried under vacuum over phosphorus pentoxide. This material proved to be inorganic in nature in that dialysis against distilled water gave no non dialyseable carbohydrate material.

Specific rotation of the starch.

Starch (ca.100mg.) was dissolved in sodium hydroxide solution (10ml.; 1N.) by shaking continuously for four hours. The resultant solution which passed easily through a number 4 sintered glass crucible, had a slight opalescence. This solution had to be diluted considerably with sodium hydroxide (1N.) before readings could be obtained using a 2 dcm. tube. The optical rotation as measured was 0.26° and the concentration of the solution as determined by acid hydrolysis was 0.875mg./ml. This is equivalent to $[\alpha]_D$ = + 149°.

Chain length of the starch.

Starch (59.2mg.) previously dried under vacuum over phosphorus pentoxide at 60°C. for 48 hours was dissolved in potassium chloride solution (40ml.; 5% W/v) with /

with the aid of heat. The solution was adjusted to pH 6 by the addition of one drop 0.1N. sodium hydroxide solution. To this solution was added sodium metaperiodate solution (10ml.; 8%^{v}/v) and the whole solution set aside in the dark on rollers. Aliquots (20ml.) were removed after 200 and 300 hours, and the formic acid release determined using 0.002N. sodium thiosulphate.

Time (hours)	200	300
CL.	34	31

β-Amylolysis limit of the starch.

Starch (ca. 35mg.) was dissolved in cold potassium hydroxide solution (5ml.; 1N.) by continuous shaking for several hours. The solution was neutralised with hydrochloric acid (1N.) and then water (10ml.) added. The polysaccharide content of this solution (1.86mg./ml.) was determined by acid hydrolysis of aliquots. Aliquots (5ml.) were used in making up the digests using both the β-amylase preparations as described in the General Methods section giving digests with a final volume of 20ml.

Aliquots (5ml.) were removed after 24 and 48 hours and the maltose content estimated against appropriate digest blanks using the Shaffer Hartman reagent.

β-amylolysis limits %	24hours	48 hours
Wallerstein enzyme	54.2	57.9
Worthington enzyme	57.0	57.6

DISCUSSION.

The study of protozoal polysaccharides present difficulties in their isolation and purification as well as imposing the necessity of small scale manipulation. In this work a simple technique has been employed for the isolation of the polysaccharides using ultrasonic vibrations for cell disruption followed by enzymic digestion of the proteinaceous material. This has provided a polysaccharide of high purity with the minimum of manipulation and the avoidance of techniques involving the possible degradation of the polysaccharide.

Characterisation of the polysaccharide has shown it to have many of the properties of a two component starch. In common with the starches isolated from other unicellular organisms, it has a lower amylose content than land plant starches shown by spectrophotometric and potentiometric measurement of iodine binding power. A comparison of the blue value with graph produced by Anderson and King (104) of blue value versus percentage amylose of various starches shows that the starch contains 14% amylose in close agreement with the value of 15.9% obtained by potentiometric iodine titration. The properties of the starch are shown below.

Glucose content, %	96
Specific rotation (in alkali)	149°
β-Amylolysis limit, %	58
CL.	~~36~~ 31
Blue value	0.244
Amylose content,%	15.9

Despite /

Despite the failure to fractionate the polysaccharide there seems little doubt of the presence of two components. The most logical explanation for the lack of fractionation seems to be that the starch is insoluble in boiling water, even for periods up to two hours, and what has been taken for an opalescent solution of the polysaccharide was in fact an extremely fine suspension of the granules which could pass readily through a number 2 sintered glass disc and which took over twenty-four hours to settle under gravity. This insolubility in water, of a protozoal starch was noted by Bourne, Stacey and Wilkinson (101) for the starch isolated from Polytomella caeca which they succeeded in dissolving only by the addition of alkali to a hot alcoholic suspension of the starch. It does seem surprising however that pretreatment of the starch with liquid ammonia, which readily gave a clear solution of the starch in a small volume of liquid, and which should have facilitated fractionation of the starch, still provided no separation of the components. Solution of the starch in hot alkali under nitrogen may enable the starch to be fractionated by normal techniques. Failing this, dimethyl sulphoxide pretreatment (111) or amylopectin precipitation with aluminium hydroxide (112) may give the desired separation.

Assuming that the amylose component of the starch has a β-amylolysis limit of 100% it can be calculated that the β-amylolysis limit of the amylopectin fraction is 55%. The /

TABLE 9.

Comparison of the properties of the starch from Polytoma uvella with starches from other sources.

	Chilomonas paramaecium	Polytomella coeca	Polytoma uvella	Potato starch	Waxy maize starch
Whole starch					
$[\alpha]_D$ in N.NaOH	+157°	+160°	+149°	+159°	+155°
Amylose content (%)	45	13 - 16	15	20	-
Blue value	0.54	0.36	0.24	0.4	-
Amylopectin					
β-Amylolysis limit (%)	60	48	55	61	54
$\overline{CL}$	22	-	26	22	24

The chain length can be calculated to be 26 . A comparison of the properties of the starch with other protozoal starches and with potato and waxy maize starch is shown opposite (Table 9). The properties show that Polytoma uvella starch is comparable with land plant starches and with other protozoal starches in its properties, although the specific rotation of the starch appears to be slightly low. This may be connected with the difficulty in obtaining a true solution of the starch.

SECTION 6.

The iodine staining of starch-type polysaccharides.

INTRODUCTION.

The intense blue colour which starch forms with iodine has long been used for the detection of starch-type compounds. The interaction of starch and iodine also forms the basis for the determination of the amylose contents of starches both by spectrophotometric estimation (113) and by potentiometric titration (35,36). The activity of preparations of branching and de-branching enzymes which effect the formation or breakdown of the α-1,6-glucosidic interchain linkages in starch-type polysaccharides (114,115) are assayed by the change in the optical density of the substrate - iodine complex in solution.

There is some evidence that the iodine staining power of amylose is related to its chain length (116,117) provided that this does not exceed a certain critical value. An attempt has also been made to relate the iodine staining properties of amylopectin and glycogen with the structure of these polysaccharides (58). There is a marked difference between the iodine staining properties of amylopectins and glycogens. Amylopectin has a maximum absorption at 530 - 550mμ, glycogen at 420 - 490mμ while the maximum optical density ranges are 0.8 - 1.2 and 0.1 - 0.4 respectively. In aqueous solutions there does not appear to be any direct relationship between the branching characteristics of these polysaccharides /

polysaccharides and their iodine staining properties. In solutions of half saturated ammonium sulphate however there does appear to be a relationship between the wavelength of maximum absorption (λ max.) and the branching properties.

Although it has been appreciated (119) that salts other than ammonium sulphate may exercise an effect on the iodine staining power of polysaccharides, a direct comparison of the effects of various salts, under conditions in which an iodine salt blank was included in the system, does not appear to have been carried out.

The iodine staining properties of amylose (120, 121) and amylopectin (122), though not glycogen, in the ultra-violet region of the spectrum have been described. In each case the measurements appear to have been carried out using a water blank whereas the inclusion of an iodine blank would have enabled a ready assessment of the spectra of the polysaccharide-iodine complexes. A direct comparison of the ultra-violet spectra of amylopectin and glycogen iodine complexes is of interest in view of the differences in the visible spectra of the two polysaccharides.

The aim of the present work has been to demonstrate the effects of various salts on the iodine staining properties of glycogens and to investigate the ultra-violet absorption spectra of the polysaccharide-iodine complexes of amylopectin and glycogen.

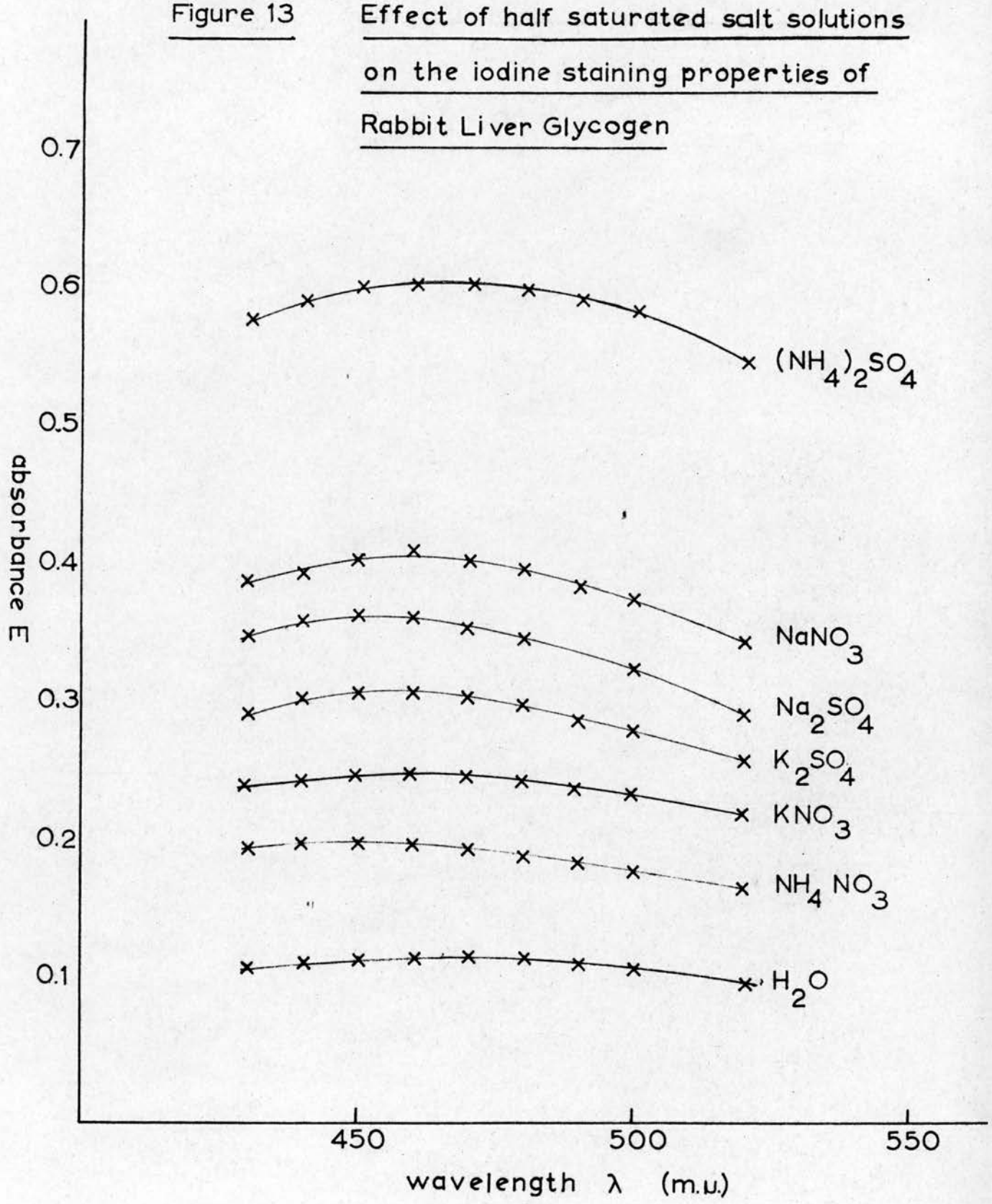
There does not appear to be a report of the effect

of /

of an amylose impurity on the wavelength of maximum absorption of an amylopectin-iodine solution and an experiment is described demonstrating the effect.

Part of the work described in this section has been published as part of a paper by Archibald, Fleming, Liddle, Manners, Mercer, and Wright, J.C.S., (1961), 1183 - 1190.

Figure 13 Effect of half saturated salt solutions on the iodine staining properties of Rabbit Liver Glycogen



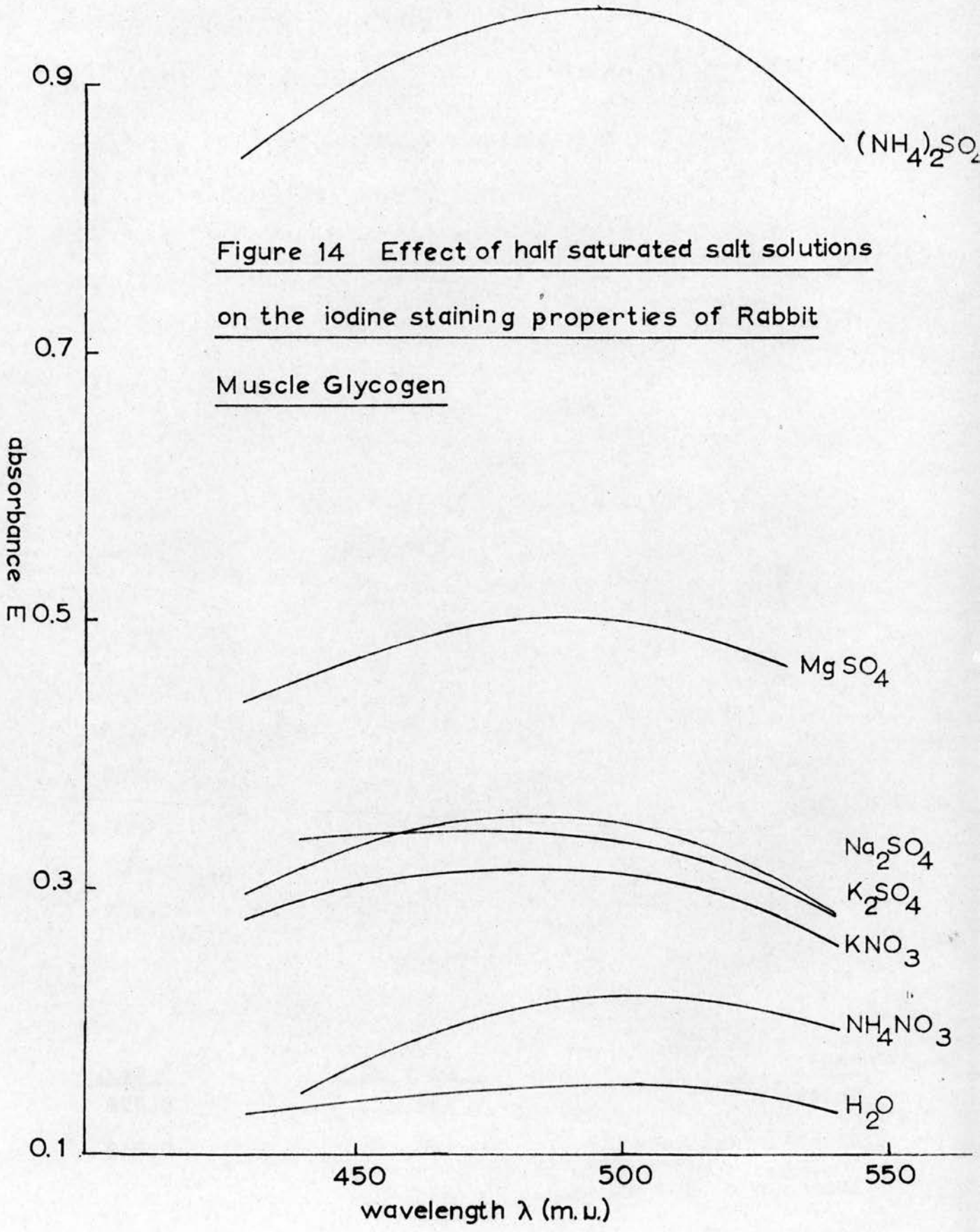


Figure 14 Effect of half saturated salt solutions on the iodine staining properties of Rabbit Muscle Glycogen

EXPERIMENTAL.

1) The effect of salts on the iodine-staining properties of glycogens.

The iodine-staining properties of three glycogens, Rabbit liver X, Rabbit muscle III, and *Mytilus edulis* VI, have been measured in various half-saturated salt solutions as described in Section 2. The results obtained follow from Tables 10, 11 and 12 and from Figures 13, 14 and 15.

TABLE 10.

Effect of half-saturated salt solutions on the iodine-staining properties of Rabbit liver X glycogen.

Conditions	λ max. (mμ.)	E max.
Water	470	0.241
Ammonium sulphate	465	0.610
Ammonium nitrate	445	0.206
Sodium sulphate	450	0.370
Sodium nitrate	460	0.418
Potassium sulphate	455	0.315
Potassium nitrate	455	0.254
Magnesium sulphate	445	0.569
Calcium chloride	440	0.359

TABLE 11.

Effect of half-saturated salt solutions on the iodine-staining properties of Rabbit muscle III glycogen.

Conditions	λ max. (mμ.)	E max.
Water	495	0.296
Ammonium sulphate	490	0.940

Ammonium /

Figure 15 Effect of half saturated salt solutions on the iodine staining properties of Mytilus edulis glycogen

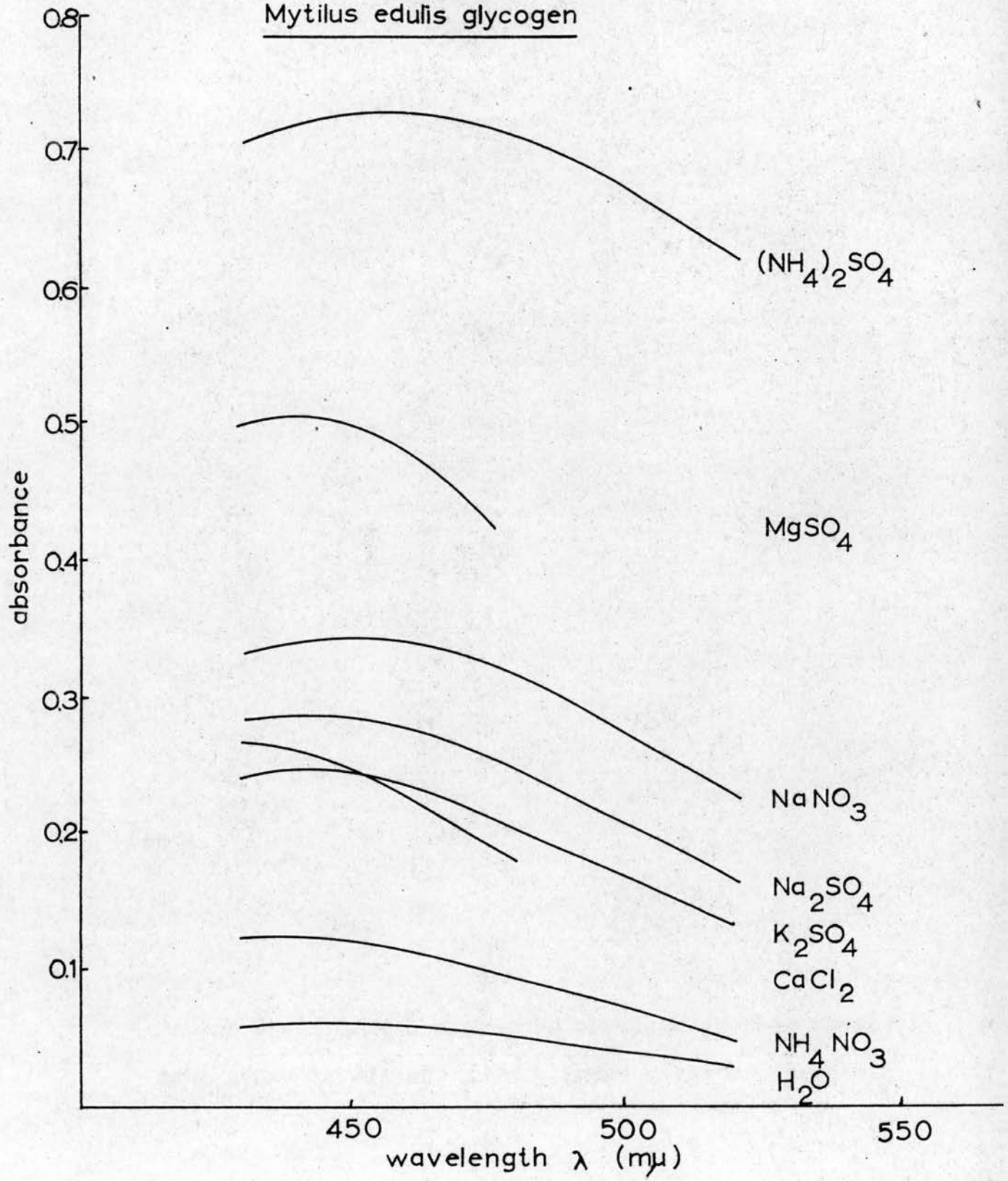


TABLE 11. (Continued)

Conditions	λ max. (mμ.)	E max.
Ammonium nitrate	505	0.215
Sodium sulphate	485	0.346
Sodium nitrate	-	-
Potassium sulphate	485	0.307
Potassium nitrate	465	0.338
Magnesium sulphate	495	0.494

TABLE 12.

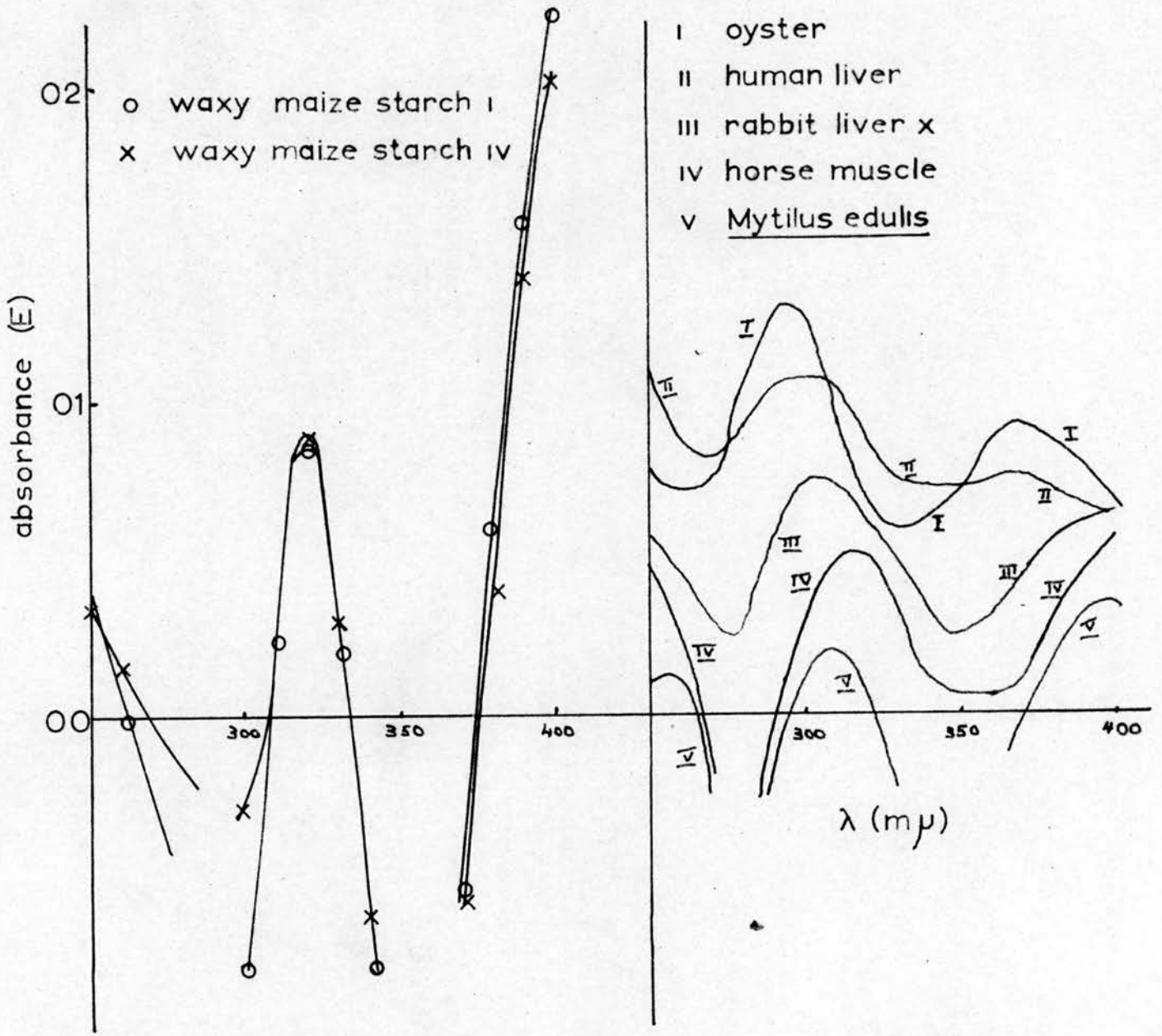
Effect of half-saturated salt solutions on the iodine-staining properties of Mytilus edulis VI glycogen.

Conditions	λ max. (mμ.)	E max.
Water	445	0.121
Ammonium sulphate	455	0.732
Ammonium nitrate	445	0.125
Sodium sulphate	445	0.285
Sodium nitrate	450	0.345
Potassium sulphate	445	0.246
Potassium nitrate	-	-
Magnesium sulphate	440	0.507
Calcium chloride	430	0.266

2) The ultra-violet spectra of amylopectin- and glycogen-iodine complexes.

Polysaccharide (25mg.) previously dried under vacuum over phosphorous pentoxide for 48 hours was dissolved in water (25ml.) with the aid of heat. An aliquot /

Figure 16 Ultra-violet spectra of amylopectin and glycogen iodine complexes



aliquot of this solution (5ml.) was pipetted into a standard flask (25ml.) along with iodine solution (0.5ml.; 0.2% W/v iodine; 2.0% W/v potassium iodide) and the solution made up to the mark with distilled water.

All solutions were measured against an appropriate iodine-potassium iodide blank in calibrated 1cm. silica cells between 250mμ and 400mμ in a Unicam SP 500 spectrophotometer. The results obtained are shown in Table 13 and Figure 16.

TABLE 13.

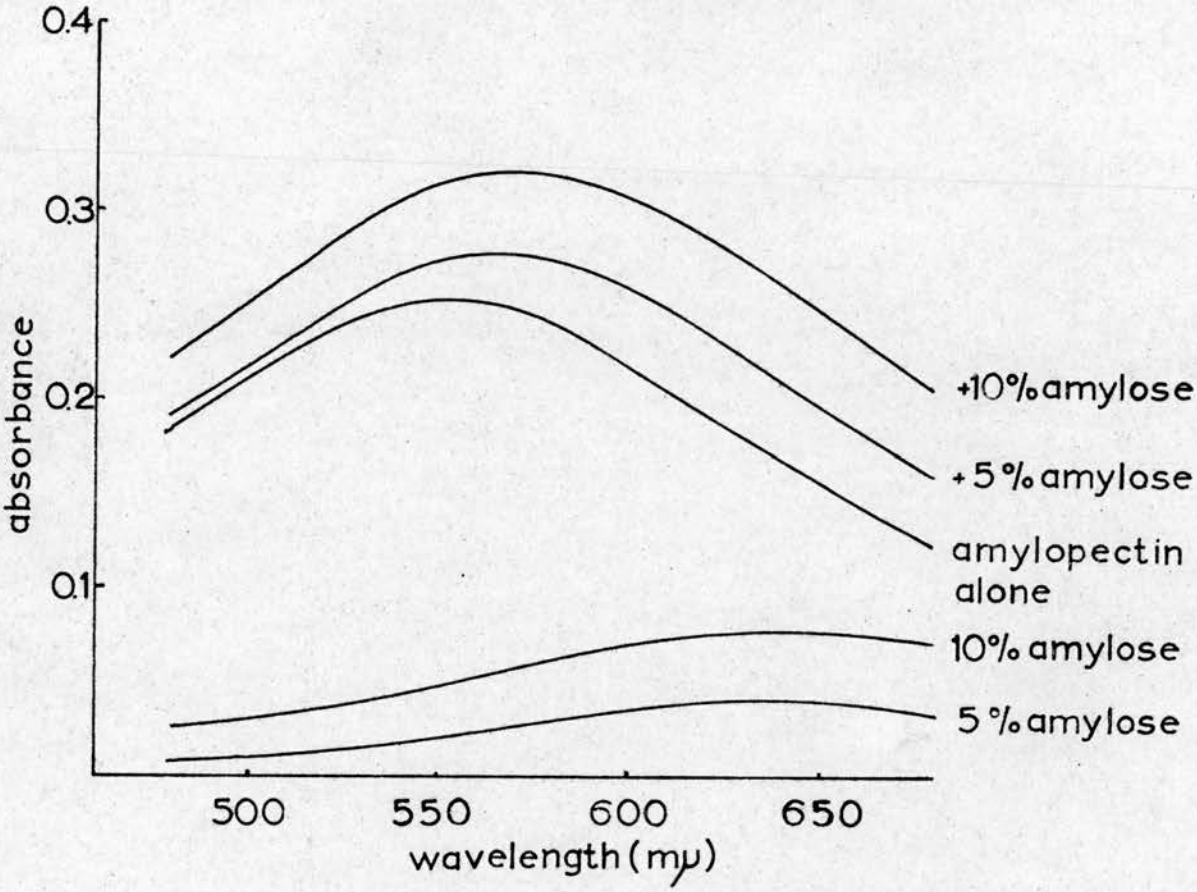
Ultra-violet spectra of amylopectin- and glycogen - iodine.complexes.

Polysaccharide	E max.	max. (mμ)	$\overline{CL}$.	$\overline{ECL}$.	$\overline{ICL}$.
Waxy maize starch I	0.085	320	22	14-15	6-7
Waxy maize starch IV	0.089	320	22	15	6
Horse muscle glycogen	0.051	315	17	11-12	4-5
Human liver glycogen	0.111	295	14	9	4
Mytilus edulis glycogen VI	0.021	305	13	8 -9	3-4
Rabbit liver glycogen X	0.077	305	12	8-9	3-5
Oyster glycogen	0.125	295	10	5-6	6-7

3) The effect of amylose on the iodine-staining properties of amylopectin.

Potato amylopectin (var. Ct. Scot) (25.5mg.) was dissolved in water (50ml.) with the aid of heat. Potato amylose (var. Ct. Scot) (5.5mg.) was dissolved in water (100ml.) with the aid of heat. To each of three standard flasks (100ml.) was added amylopectin solution (5ml.) and iodine /

Figure 17 Effect of amylose on the iodine-staining properties of amylopectin



iodine solution (5ml.;0.2%^{W}/v iodine; 2.0% $w/_{v}$ potassium iodide). To one flask was added amylose solution (2.5ml.) and to a second amylose solution (5ml.) was added. All three flasks were made up to the mark with distilled water. To two standard flasks (100ml.) were added amylose solution (2.5ml. and 5ml. respectively) and iodine solution (5ml.). The flasks were then made up to the mark with distilled water. The optical density of all five flasks was measured between 480mμ and 680mμ against an appropriate iodine blank in a Unicam SP 500 spectrophotometer. The results obtained follow from Table 14 and Figure 17.

TABLE 14.

Effect of amylose on the iodine-staining properties of amylopectin.

	λmax.	E max.
Amylopectin	555	0.256
Amylopectin +5% amylose	561	0.280
Amylopectin + 10% amylose	568	0.326
Absorption of 5% amylose at 561mμ	-	0.026
Absorption of 10% amylose at 568mμ	-	0.061

DISCUSSION.

The effect of salts on the iodine-staining properties of glycogens is principally to increase the intensity of the colour of the glycogen iodine complex while effecting small changes in the wavelength of maximum absorption (λ max.) of the complex. The increase in the intensity, of the salts studied, is greatest with ammonium sulphate followed by magnesium sulphate and ammonium nitrate. The reason for this increase has been suggested by Schlamowitz (118) to be that the helical structure postulated for the polysaccharide iodine complex is relatively free of election donor groups and the salt acts by removing occluded water, which has electron donor properties, from the helix.

TABLE 15.

Solubility of salts compared with the effect of their half-saturated solutions on the iodine-staining properties of Rabbit liver X glycogen.

Salt	Solubility (moles/litre)	E max.
Ammonium sulphate	5.8	0.61
Magnesium sulphate	2.8	0.57
Sodium sulphate	0.8	0.37
Potassium sulphate	0.7	0.32
Ammonium nitrate	1.3	0.21
Sodium nitrate	8.8	0.42
Potassium nitrate	3.1	0.25
Calcium chloride	5.4	0.36
Water	-	0.12

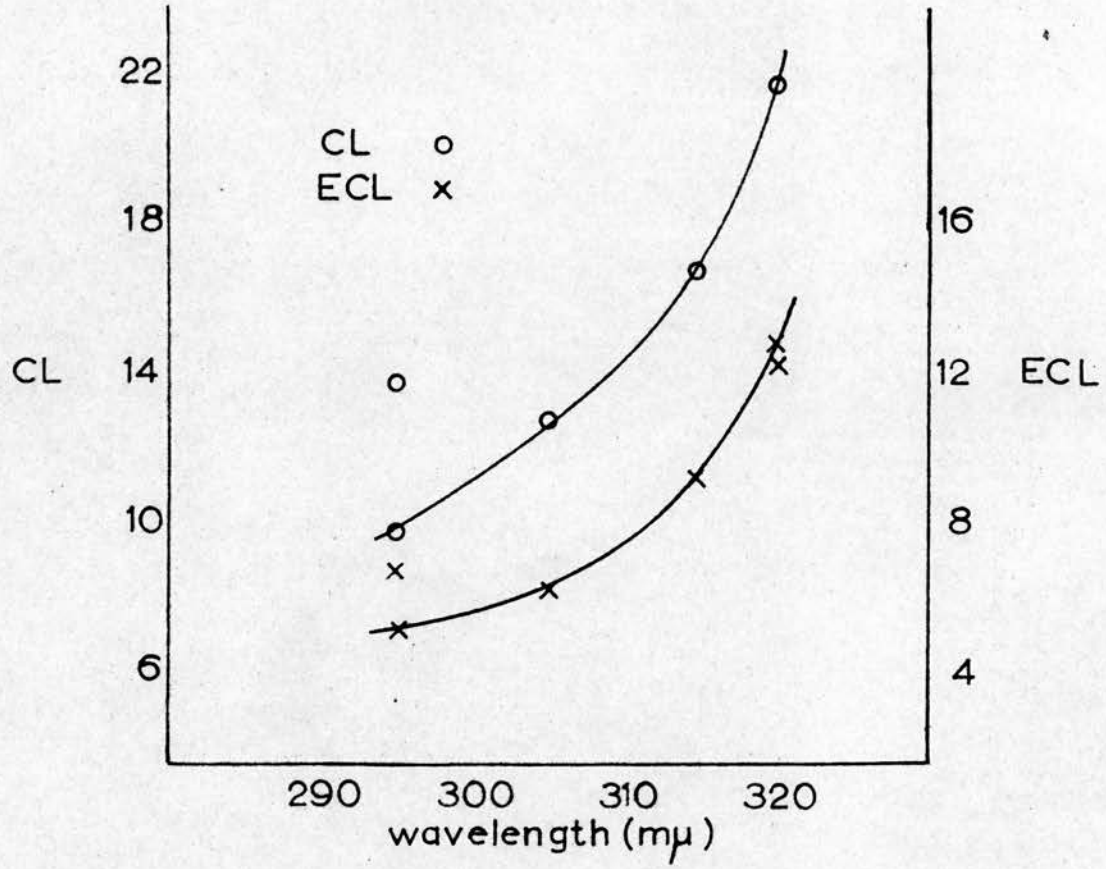
Why ammonium sulphate should be more effective

than /

than the other salts tested is not certain. The solubilities of the various salts used in this work, measured as the weight of the salt soluble in 100ml. water, gives an approximate molarity for the saturated solutions. Comparison of these values with the optical density of the iodine stain of one of the glycogens studied (Table 15) shows that there is no direct comparison possible between molarity of salt and iodine staining power. There may however be two separate series, one for nitrate salts and one for sulphate salts and it may be that solutions of salts of constant molarity with the same anion will have similar effects on the iodine-staining properties of glycogen.

Study of the ultra-violet spectra of the polysaccharide-iodine complexes of amylopectins and glycogens has shown that the shape of the curves obtained are roughly similar for both the groups of polysaccharides and that there is no apparent feature which could sharply differentiate an amylopectin from a glycogen. Difficulties were encountered in the measurement of the spectra owing to the high absorption of the iodine blank solution and in future work it may prove more convenient to increase the polysaccharide-iodine ratio while reducing the iodine concentration. The absorption peak occurring between 290mμ and 330mμ is very sharp and the position of its apex, which /

Figure 18 Correlation of CL. and ECL. with iodine-staining properties



which appears to bear some relation to $\overline{CL}$ or $\overline{ECL}$ of the polysaccharides (See Figure 18), can no doubt be determined to $\pm$ 1mμ, in contrast to the broad flat peak of glycogens in the visible region of the spectrum which has already been used in an attempt to correlate glycogen structure with iodine staining power.

The effect of an amylose impurity on the iodine staining properties of an amylopectin iodine complex is to increase the maximum absorption and the wavelength of maximum absorption slightly. This effect is sufficiently small as to have a negligible effect on the iodine staining properties of amylopectins isolated from starches by the accepted techniques of thymol and butanol fractionation.

BIBLIOGRAPHY.

1) Bourne, Stacey, and Wilkinson, J.C.S., (1950), 2694.

2) Eddy, Fleming, and Manners, J.C.S., (1958), 2827.

3) Carlson and Hehre, J.B.C., (1949), 177, 281.

4) Whistler and Doane, Cereal Chem., (1961), 38, 251.

5) Peat, Pirt, Thomas, and Whelan, J.C.S., (1952), 705.

6) Cunningham, Manners, Wright, and Fleming, J.C.S., (1960), 2602.

7) Baum, Gilbert, and Scott, Nature, (1956), 177, 889.

8) Cowie, Fleming, Greenwood, and Manners, J.C.S., (1958), 697.

9) Posternak, J.B.C., (1951), 188, 317.

10) Peat, Thomas, and Whelan, J.C.S., (1952), 722.

11) Kjölberg and Manners, Biochem. J., (1962), 84, 50P.

12) Brown, Dunstan, Halsall, Hirst, and Jones, Nature, (1945), 156, 785.

13) Wolfrom, Tyree, Galkowski, and O'Neil, J.A.C.S., (1951), 73, 4927.

14) Barker, Hirst, and Young, Nature, (1941), 147, 296.

15) Meyer, and Bernfield, Helv. Chem. Acta, (1940), 23, 875.
Meyer, and Fuld, ibid, (1941), 24, 375.

16) Larner, Illingworth, Cori, and Cori, J.B.C., (1952) 199, 641.

17) Partridge, and Westall, Biochem. J., (1948), 42, 238.

18) Partridge, Symposium Biochem. Soc., (1949), 3, 57.

19) Timell, Glaudemans, and Currie, Anal. Chem., (1956), 28, 1916.

20) Bonner, Chem. and Ind., (1960), 545.

21) Sampson, Schild, and Wicker, Chem. and Ind., (1961) 82.

22) Lemieux, and Bauer, Anal. Chem., (1954), 26, 920.

23) Benvenue, and Williams, Arch. Biochem. Biophys. (1951), 34, 225.

24) Whistler and Durso, J.A.C.S., (1950), 72, 677.

25) Hughes and Whelan, Chem. and Ind., (1958), 884.

26) Somogyi, J.B.C., (1952), 195, 19.

27) Shaffer and Hartman, J.B.C., (1921), 45, 365.

28) Hobson, Whelan and Peat, J.C.S., (1950), 3570.

29) Walker and Whelan, Biochem. J., (1960), 76, 264.

30) Consden and Stanier, Nat., (1952), 169, 783.

31) Lindberg and Swan, Acta, Chem. Scand., (1960), 14,1043.

32) Foster, Chem. and Ind., (1952), 1050.

33) Aspinall and Ferrier, Chem. and Ind., (1957), 1216.

34) Hudson and Johnson, J.A.C.S., (1917), 39, 1274.

35) Hudson, Schoch and Wilson, J.A.C.S., (1943), 65,1380.

36) Rundle, French and Bates, J.A.C.S., (1943), 65, 142.

37) Peat, Whelan, Turvey and Morgan, J.C.S., (1961), 623.

38) Woolf, Nat., (1953), 171, 841.

39) Hough, Jones and Richards, Chem. and Ind., (1953),1064.

40) Diedrich, and Anderson, Anal. Biochem., (1961), 2, 68.

41) Malaprade, Bull. Soc. Chim. (France),(1928), 43, 683.

42) Bobbitt, Adv. Carbohydrate Chem., (1956), 11, 1.

43) Hirst, Jones and Roudier, J.C.S., (1948), 1779.

44) Alexander, Dimler and Mehltretter, J.A.C.S., (1951), 73, 4658.

45) Hamilton and Smith, J.A.C.S., (1956), 78, 5910.

46) Goldstein and Smith, J.A.C.S., (1960), 82, 3421.

47) Guthrie, Adv. Carbohydrate Chem., (1961), 16, 138.

48) Jackson, J.C.S., (1900), 77, 129.

49) Anderson, Greenwood and Hirst, J.C.S., (1955), 225.

50) Bell and Manners, J.C.S., (1954), 1891.

51) Gibbons and Boissonnas, Helv. Chim. Acta, (1950), 33, 1477.

52) McWilliam and Percival, J.C.S., (1951), 2259.

53) Greenwood and Robertson, J.C.S., (1954), 3769.

54) Hughes and Nevell, Trans. Farraday Soc., (1948) 44, 941.

55) Dubois, Gilles , Hamilton, Rebers and Smith, Anal. Chem., (1956), 28, 350.

56) Chanda, Hirst, and Manners, J.C.S., (1957), 1951.

57) Brown, Halsall, Hirst and Jones, J.C.S., (1948), 27.

58) Archibald, Fleming, Liddle, Manners, Mercer and Wright, J.C.S., (1961), 1183.

59) Hough and Perry, Chem. and Ind., (1956), 768.

60) Head and Hughes, J.C.S., (1954), 603.

61) Perlin, J.A.C.S., (1954), 76, 4101.

62) Manners and Wright, J.C.S., (1961), 2681.

63) Pascu and Mora, J.A.C.S., (1950), 72, 1045.

64) Kent, Biochem. J., (1953), 55, 361.

65) Thompson, Anno, Wolfrom and Inatome, J.A.C.S., (1954), 76, 1309.

66) Peat, Whelan, Edwards, and Owen, J.C.S., (1958), 586.

67) Wolfrom, Schuetz, and Cavalieri, J.A.C.S., (1948), 70, 514.

68) Thompson, Wolfrom and Quinn, J.A.C.S., (1953), 75, 3003.

69) Thompson and Wolfrom, J.A.C.S., (1956), 78, 4116.

70) Bacon and Bacon, Biochem. J., (1954), 58, 396.

71) Peat, Turvey and Evans, J.C.S., (1959), 3223.

72) Thompson and Wolfrom, J.A.C.S., (1957), 79, 4212.

73) Barker, Bourne and Stacey, J.C.S., (1953), 3084.

74) Gakhokidze, J. Gen. Chem. U.S.S.R., (1946), 16, 1923. Chem. Abs., (1947), 41, 6210b.

75) Barker, Bourne, O'Mant and Stacey, J.C.S., (1957) 2448.

76) Haq and Whelan, J.C.S., (1958), 1342.

77) Pazur and Budovitch, J.A.C.S., (1956), 78, 1885.

78) Pazur and French, J.B.C., (1952), 196, 265.

79) Jones, Perry and Stoodley, Can. J. Chem. (1962), 40, 856.

80) Greenwood and Thompson, J.C.S., (1962), 222.

81) Wallenfels, Naturwiss, (1950), 37, 491.

82) Bell, Manners and Palmer, J.C.S., (1952), 3760.

83) Barker, Bourne, Grant and Stacey; Haq and Whelan, Nature, (1956), 178, 1221; 1222.

84) Park and Johnson, J.B.C., (1949), 181, 149.

85) Duncan and Manners, Biochem. J., (1958), 69, 343.

86) French and Wild, J.A.C.S., (1953), 75, 2612.

87) Peat, Whelan, Turvey and Morgan, J.C.S., (1961), 623.

88) Fleming, Hirst and Manners, J.C.S., (1956), 2831.

89) Banks, Meinwald, Rhind-Tutt, Sheft and Vernon, J.C.S., (1961), 412. also p. 3240 !

90) Peat, Turvey and Evans, J.C.S. (1959), 3341.

91) Ough, Anal. Chem. (1962), 34, 660.

92) Peat, Roberts and Whelan, Biochem. J., (1952), 51, xvii.

93) Radomski and Smith, Cereal Chem., (1962), 39, 30.

94) Whelan and Roberts, Nature, (1952), 170, 748.

95) Wise, Dimler, Davis and Rist, Anal. Chem., (1955), 27, 33.

96) Bernfeld, Methods in Enzymology (Ed. Colowick and Kaplan; Academic Press, New York.) Volume 1, p. 150.

97) Fischer and Stein, Arch. Sci., (1954), 7, 131.

98) Manners and Wright, J.C.S., (1962), 1597.

99) Challinor, Haworth and Hirst, J.C.S., (1934), 1562.

100) Fogg, Metabolism of Algae, Methuen & Co., London.

101) Bourne, Stacey and Wilkinson, J.C.S., (1950), 2694.

102) Eddy, Fleming and Manners, J.C.S., (1958), 2827.

103) Archibald, Hirst, Manners and Ryley, J.C.S., (1960), 556.

104) Anderson and King, J.C.S., (1961), 2914.

105) Fisher and Percival, J.C.S., (1957), 2666.

106) McKinnell and Percival, Unpublished results.

107) Brechot, Compt. rend. Soc. Biol., (1937), 126, 555.

108) Pringsheim, Naturwiss., (1935), 23, 120.

109) Clark and Stone, Biochim. Biophys. Acta, (1960) 44, 61.

110) Sevag, Lackmann and Smolens, J. Biol. Chem., (1938), 124, 425.

111) Killion and Foster, J. Polymer Sci., (1960), 46, 65.

112) Hobson, Pirt, Whelan and Peat, J.C.S., (1951), 801.

113) Bourne, Haworth, Macey and Peat, J.C.S., (1948), 924.

114) Hobson, Whelan and Peat, J.C.S., (1951), 1451.

115) Kjölberg and Manners, Biochem. J., in the press.

116) Baldwin, Bear and Rundle, J.A.C.S., (1944), 66, 111.

117) Swanson, J.B.C., (1948), 172, 825.

118) Schlamowitz, J.B.C., (1951), 190, 519.

119) Kuge and Ono, Bull. Chem. Soc. Jap., (1960), 33, 1269.

120) Kontos, Dissertation Abs., (1960), 21, 1078.

121) Thoma, and French, J.A.C.S., (1960), 82, 4144.

122) Bebington, Bourne and Wilkinson, J.C.S. (1952), 246.

Preprinted from the Journal of the Chemical Society, March, 1961, (232), pages 1183—1190.

232. α-1,4-*Glucosans. Part XI.*[1] *The Absorption Spectra of Glycogen– and Amylopectin–Iodine Complexes.*

By A. R. Archibald, I. D. Fleming, A. Margaret Liddle, D. J. Manners, G. A. Mercer, and A. Wright.

The absorption spectra of the iodine complexes of a large number of starch-type polysaccharides have been measured. Amylopectins and glycogens show maximum absorption at *ca.* 540 and *ca.* 460 mμ respectively. This dissimilarity may be correlated with differences in type of iodine-binding arising from variations in the average length of the interior chains of the two polysaccharide-types. Mammalian glycogens are more iodophilic than invertebrate glycogens; this fact cannot at present be related to known structural features.

Addition of ammonium sulphate and other salts to a polysaccharide–iodine solution causes a marked increase in the iodine-staining power of glycogen, but only a small increase with amylopectin. Under these conditions, the position of maximum absorption is approximately related to the degree of branching in the polysaccharide.

The action of branching or debranching enzymes, which catalyse the synthesis or hydrolysis of α-1,6-glucosidic inter-chain linkages in starch-type polysaccharides may be followed by measurement of changes in the iodine-staining power of the substrate. For example, the activity of liver branching enzyme is determined from the decrease in optical density at 570 mμ of an amylopectin–iodine solution,[2] whilst R-enzyme is assayed from the increase in " blue value " of amylopectin β-dextrin.[3] There is some evidence [4,5] that the iodine-staining power of amylose is related to the average chain length ($\overline{CL}$) or degree of polymerisation ($\overline{DP}$) provided that the amylose molecules do not exceed a certain critical size, although there is no apparent agreement on the actual value of this. However, information on the possible relation between the absorption spectrum of a glycogen– or amylopectin–iodine complex and the degree of branching in the polysaccharide is not available, and we now report such an investigation. A preliminary account of part of this work has been given.[6]

In previous papers of this Series,[1,7,8] the $\overline{CL}$ and β-amylolysis limit of a large number of glycogens and amylopectins, have been reported; from these data, the relative lengths of the exterior and the interior chains have been calculated. The absorption spectra of the iodine complexes of these polysaccharides have now been determined, in conditions similar to those used by Peat and his co-workers [9] in their iodine-staining studies of the action of R-enzyme on glycogen, *i.e.*, the light absorption in the range 400—700 mμ of a solution containing 0·01% of polysaccharide and 0·02% of iodine in 0·2% aqueous potassium iodide was measured on a Unicam S.P. 500 or S.P. 600 spectrophotometer against an iodine–iodide reference solution. Typical curves are shown in Fig. 1; effects due to light absorption by the polysaccharide alone are negligible under these conditions. With glycogens, a wide absorption peak covering 20—30 mμ was frequently obtained, and the $\lambda_{max.}$ quoted represent the mid-points; with amylopectins, a sharper peak was observed and the $\lambda_{max.}$ values are significant to ±5 mμ. From the above curves $\lambda_{max.}$ and $E_{max.}$ (the extinction or

absorption value at this wavelength) were noted for each polysaccharide, and the results are recorded in Table 1. Control experiments showed that the $\lambda_{max.}$ values were unaffected by variation in the polysaccharide : iodine ratio; the figures as quoted for $E_{max.}$ values are considered to be significant, although slight variation was observed (*e.g.*, in triplicate solutions Floridean starch II had $E_{max.}$ 0·68, 0·67, and 0·72 at 530 mμ).

The general results indicate a marked difference in the absorption spectra of the iodine complexes of amylopectins and glycogens. The former complexes show much stronger absorption ($\lambda_{max.}$ 530—550 mμ, $E_{max.}$ 0·8—1·2) than those of glycogen ($\lambda_{max.}$ 420—490 mμ, $E_{max.}$ 0·1—0·4) even though the $\overline{CL}$ values of some amylopectins (*ca.* 20) are not greatly different from those of certain glycogens. This is especially true of malt amylopectin and horse muscle glycogen (Fig. 1).

The iodine absorption spectra of individual glycogens vary considerably and, for aqueous solutions, they appear to depend upon the biological source and not on the average or exterior chain length. There is no correlation between the $\lambda_{max.}$, $E_{max.}$, or extinction at a particular wavelength (*e.g.*, 460 mμ) and the branching characteristics. It follows that

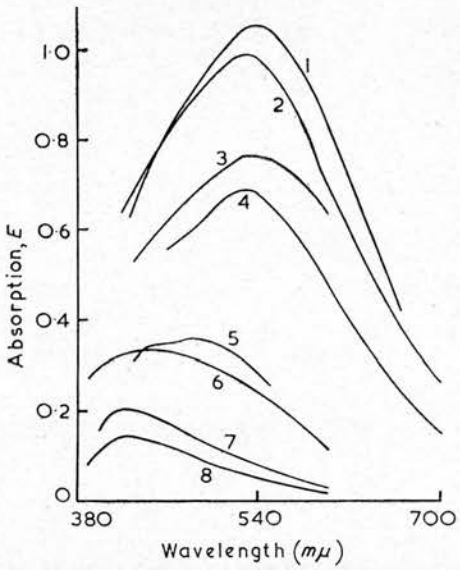


FIG. 1. *Light-absorption curves of iodine-stained polysaccharides.*

1, Waxy sorghum starch; 2, waxy maize starch; 3, malt amylopectin; 4, Floridean starch; 5, horse muscle glycogen; 6, rabbit liver glycogen; 7, skate liver glycogen; 8, *Mytilis edulis* glycogen.

increases in the absorption spectra of the iodine complexes of liver glycogen isolated from animals in different metabolic conditions [10] are not necessarily due to changes in molecular structure. Previous observations [11] that fish and invertebrate glycogens give yellow-brown stains with iodine whilst mammalian samples give red-brown colours have been confirmed. In general, the iodine-staining power increased in the order, glycogen β-limit dextrin, invertebrate glycogen, mammalian liver glycogen, and mammalian muscle glycogen. This variation of iodine-staining power with biological source is an important factor in iodine-staining methods for the determination of glycogen, and separate calibration curves are suggested for glycogen samples from different species.[12]

The iodine-staining of the Floridean starches and sweet-corn polysaccharides is of special interest. The algal polysaccharides resemble typical glycogens rather than amylopectins with regard to degree of branching,[7] and yet give appreciably more intense iodine-stains than do the animal polysaccharides. A sample of Floridean starch examined by Peat, Turvey, and Evans [13] which had $\overline{CL}$ 15 also showed $\lambda_{max.}$ 530 mμ. The water-soluble polysaccharides from *Zea mays* are structurally indistinguishable from animal glycogens,[14] and the data in Table 1 are in accord with this finding.

In contrast to the glycogens, amylopectins from various plant and protozoal starches

showed similar $\lambda_{max.}$ values; there was some variation in the extinction at 680 mμ and this is attributed to the presence of small amounts of amylose as impurity in the amylopectin samples. For example, although waxy maize starch is normally considered to be free from amylose, potentiometric titration [15] has indicated the presence of 1·4% of linear

TABLE 1. *Iodine-staining properties of polysaccharides.*

Polysaccharide	$\lambda_{max.}$ (mμ)	$E_{max.}$	$\overline{CL}$ *	$\overline{ECL}$ †	$\overline{ICL}$ †
Glycogens:					
Arenicola	420	0·2	11 [e]	7—8	2—3
Ascaris lumbricoides	435	0·2	12 [a]	8	3
Cardium	420	0·1	8 [e]	3—4	3—4
Cat liver IV	465	0·3	13 [e]	9—10	2—3
Cock liver	440	0·1	13 [e]	7—8	4—5
Fœtal pig liver	440	0·5	11 [e]	8	2
Helix pomatia II	425	0·1	7 [a]	4	2
Horse muscle	460	0·3	11 [a]	7	3
Human liver I	460	0·2	14 [b]	9	4
II	430	0·05	6 [b]	2	3
Mytilus edulis I	435	0·1	12 [a]	7—8	3—4
II	440	0·2	16 [a]	10—11	4—5
IV	450	0·1	12 [e]	8—9	2—3
VII	420	0·2	13 [e]	8—9	3—4
VIII	420	0·2	13 [e]	8—9	3—4
IX	430	0·2	10 [e]	7—8	1—2
X	420	0·2	14 [e]	8—9	4—5
Oyster	440	0·2	10 [e]	6—7	2—3
Rabbit liver I	455	0·2	13 [e]	5—6	6—7
IV	460	0·3	13 [e]	8—9	3—4
V	450	0·3	14 [e]	9—10	3—4
VIII	460	0·2	13 [e]	9	3
IX	485	0·4	13 [e]	9	3
X	475	0·3	12 [e]	8—9	2—3
Rabbit muscle I	490	0·4	12 [a]	8	3
III	490	0·3	13 [e]	8—9	3—4
Skate liver	420	0·2	13 [e]	8—9	3—4
Trichomonas gallinae II	440	0·3	13 [8]	8—9	3—4
Yeast (brewer's)	430	0·3	13 [8]	8	4
Amylopectins:					
Potato I (King Edward)	540	1·3	23 [e]	14—15	7—8
II (Great Scot)	555	1·2	24 [d]	17	6
Protozoal	530	1·3	22 [6]	15—16	5—6
Waxy maize starch I	530	1·0	22 [6]	14—15	6—7
II	530	0·9	21 [6]	15	5
Waxy sorghum starch II	535	1·0	22	15	6
Other polysaccharides:					
Ascaris glycogen β-dextrin	430	0·1	6—7	2—3	3
Fœtal sheep liver glycogen β-dextrin	430	0·1	6—7	2—3	3
Floridean starch I	500	0·8	9 [7]	6—7	1—2
II	530	0·7	12 [7]	7	4
III	530	0·6	13 [7]	7—8	4—5
Helix glycogen β-dextrin	430	0·03	4—5	1—2	2
Rabbit liver glycogen dextrin	460	0·3	9	5	3
Waxy maize starch β-dextrin	530	0·9	10	2—3	6—7
Waxy sorghum starch β-dextrin	540	0·8	12	2—3	8—9
Zea mays *polysaccharides*:					
Phytoglycogen A	450	0·2	13 [14]	9	3
B	430	0·2	7 [14]	5	1
Fraction 55—60	480	0·2	10 [14]	—	—
60—65	450	0·2	11 [14]	—	—
65—70	445	0·1	10 [14]	—	—

* Average chain length, determined by periodate oxidation (superscript numbers refer to the previous results and the superscript letters to the following references: *a*, Bell and Manners, *J.*, 1952, 3641; *b*, Calderbank, Kent, Lorber, Manners, and Wright, *Biochem. J.*, 1960, **74**, 223; *c*, Manners and Wright, unpublished work; *d*, Fleming and Mercer, unpublished work; *e*, Liddle and Manners, *J.*, 1957, 3432).

† $\overline{ECL}$, exterior chain length, *i.e.*, no. of glucose residues removed by β-amylase + 2·5; $\overline{ICL}$, interior chain length, *i.e.*, $\overline{CL} - \overline{ECL} - 1$.

polysaccharide in sample I. However, control experiments have shown that the presence of even 5% of amylose impurity does not appreciably affect the position of $\lambda_{max.}$.

The iodine-staining properties of glycogen and amylopectin show a more marked difference after β-amylolysis. With glycogen, both $\lambda_{max.}$ and $E_{max.}$ are decreased, whereas amylopectin β-dextrin has the same $\lambda_{max.}$ as the original polysaccharides. It is clear that in amylopectin, the $\lambda_{max.}$ of the iodine complex is not related to the length of the exterior chains. In contrast, there is evidence [15] that the iodine-binding power of branched α-1,4-glucosans, as determined by potentiometric titration, increases with the length of the exterior chains.

TABLE 2. *Calculated * lengths of A- and B-chains.*

	$\overline{CL}$	β-Amylolysis limit (%)	Average lengths A-chain	B-chain
Amylopectin	20 †	57 †	14	26
Glycogen	12 †	45 †	8	16

* The exterior A- and B-chain stubs in a β-dextrin are assumed to contain 2—3 glucose residues.
† Typical experimental results.

The above results indicate that the nature of the iodine-binding in glycogen and in amylopectin is different. Higginbotham [16] has suggested that amylopectin binds iodine partly by the adsorption of iodine molecules or tri-iodide ions, and partly by a mechanism in which iodine molecules are arranged endwise and axially inside a series of helices of α-1,4-linked glucose residues. Each coil of the helix is believed to contain six glucose residues and one iodine molecule.[4] The smallest amylose-type molecule which gives a

FIG. 2. *Relation between interior chain length of a polysaccharide and $\lambda_{max.}$ of the iodine complex. ● and ○ represent glycogen and amylopectin respectively.*

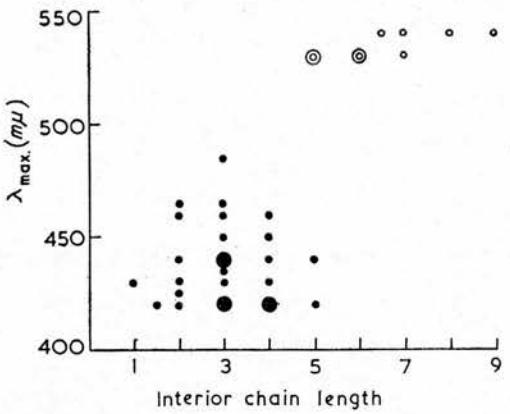
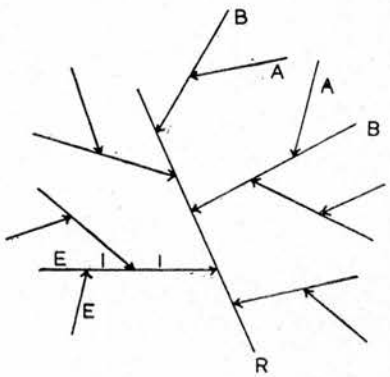


FIG. 3. *Multiply-branched structure for amylopectin and glycogen.*



R, *Reducing-group*; E, *exterior chain*; I, *interior chain*; A, A-*chain*; B, B-*chain*.

colour with iodine probably contains *ca.* 18 glucose residues,[17] *i.e.*, a sequence of three helical coils is required.

One possible explanation for the difference in the iodine-staining properties of amylopectin and glycogen is the characteristic difference in the average length of the interior chains (see Fig. 2) and hence of the B-chains.* By definition, the molecules contain an equal number of exterior and interior chains, and if we assume that amylopectin and glycogen comprise an equal number of A- and B-chains,[18] each A-chain * represents an exterior chain, whilst every B-chain contains one exterior and

* An A-chain (side-chain) is linked to the molecule only by the reducing group, whilst B-chains (main-chains) which are similarly linked, also have other chains attached to them.

two interior sections (see Fig. 3). Calculation (Table 2) shows that with amylopectin a considerable proportion of the B-chains would be of a suitable length to assume a helical configuration. In contrast, only a small proportion of the B-chains in glycogen would exceed $\overline{CL}$ 18. It has been suggested that the presence of branch points would interfere with complex formation;[4] however, inspection of models[19] of

TABLE 3. *Iodine-staining properties of glycogens determined by Schlamowitz's method.*[22]

Glycogen	$\lambda_{max.}$ (mμ)	$E_{max.}$	$\overline{CL}$ *	$\overline{ECL}$ *	$\overline{ICL}$ *
Mytilus edulis IX	500	0·5	10 [e]	7—8	1—2
Rabbit muscle I	520	0·3	12 [a]	8	3
Rabbit liver X	500	0·6	12 [f]	8—9	2—3
Tetrahymena pyriformis II	505	0·5	14 [f]	9	4
Trichomonas foetus	505	0·5	15 [f]	11—12	2—3
Trichomonas gallinae II	495	0·6	13 [f]	8—9	3—4
Zea mays					
Phytoglycogen A	550	0·5	13 [14]	—	—
B	530	0·2	7 [14]	—	—

* See Footnotes to Table 1. [f] Manners and Archibald, *J.*, 1957, 2205.

TABLE 4. *Effect of half-saturated ammonium sulphate on the absorption spectra of polysaccharide–iodine complexes.*

	Water		NH_4 sulphate				
Polysaccharide	$\lambda_{max.}$ (mμ)	$E_{max.}$	$\lambda_{max.}$ (mμ)	$E_{max.}$	$\overline{CL}$ *	$\overline{ECL}$ *	$\overline{ICL}$ *
Glycogens:							
Fœtal sheep liver	450	0·3	485	0·6	13 [a]	9	3
Horse muscle	490	0·3	495	0·6	16—17 [1]	10—11	5
Human kidney	445	0·2	460	0·6	14 [b]	9	4
Human muscle	445	0·1	460	0·6 †	11 [a]	7	3
Mytilus edulis V	420	0·2	450	0·8	9 [f]	6	2
VI	445	0·1	480	1·0	13 [f]	8—9	3—4
Ox muscle	480	0·3	505	0·7 †	15 [1]	10	4
Rabbit liver VI	465	0·3	500	1·1	18 [e]	12	5
XII	460	0·2	500	1·0	17 [e]	9—10	6—7
XIII	470	0·3	480	1·0	15 [e]	9—10	4—5
Rabbit muscle II	460	0·2	490	0·9	11 [e]	6—7	3—4
Tetrahymena pyriformis I	445	0·3	465	0·9	13 [g]	8—9	3—4
II	440	0·3	485	0·9	14 [f]	9	4
Trichomonas foetus	445	0·4	500	1·1	15 [h]	11—12	2—3
Trichomonas gallinae II	440	0·3	480	1·0	13 [f]	8—9	3—4
Amylopectins:							
Malted barley	535	0·9	540	1·1	18 [i]	10—11	6—7
Potato III (Kerr's Pink)	540	1·1	545	0·7 †	22 [c]	16	5
IV (Epicure)	550	1·3	545	0·7 †	24 [j]	16	7
Protozoal (*Chilomonas paramecium*)	540	1·2	545	1·4	22 [k]	15—16	5—6
Waxy maize starch IV	530	1·1	560	1·6	22 [c]	15	6
Waxy sorghum starch I	540	1·1	560	1·2	25 [c]	15—16	8—9
β-Dextrins:							
Fœtal sheep liver glycogen	425	0·1	415	0·4 †	6—7	2—3	3
Mytilus edulis VI	420	0·03	420	0·3 †	7	2—3	3—4
Rabbit liver II	420	0·2	425	0·5 †	9	2—3	5—6
Waxy maize starch I	535	0·9	535	0·5 †	10	2—3	6—7

* See Footnotes to Table 1; additional references are, *g*, Manners and Ryley, *Biochem. J.*, 1952, **52**, 480; *h*, *idem*, *ibid.*, 1956, **59**, 369; *i*, Aspinall, Hirst, and McArthur, *J.*, 1955, 3075; *j*, Banks and Greenwood, unpublished work; *k*, Archibald, Hirst, Manners, and Ryley, *J.*, 1960, 556.

† Polysaccharide concentration 0·005%.

glucose residues in a helix shows that the primary 6-alcohol groups are situated on the outer surfaces of the helix so that the attachment of side-chains to a B-chain should not necessarily affect the interior of the helix.

An additional factor concerns the ability of iodine molecules (size approx 8 Å) and the even larger tri-iodide ion to penetrate the interior of the compact glycogen molecule, as compared with the more open interior of an amylopectin molecule. This is illustrated by the fact that amylopectin β-dextrin, which has the same degree of branching as a normal

glycogen, still retains the characteristic amylopectin-type absorption with $\lambda_{max.}$ *ca.* 535 mμ.

The spectral differences between the iodine complexes may, therefore, be related to differences in the average distance between branch points in the interior of the molecules. Since it is now known that glycogen and amylopectin have similar degrees of multiple branching (*i.e.*, the ratios of A-chains to B-chains are similar),[18] this property is not a controlling factor (cf. ref. 15).

It is concluded that, under the above experimental conditions, the absorption spectra of polysaccharide–iodine complexes cannot be directly related to the proportion of α-1,6-glucosidic inter-chain linkages. The use of iodine-staining methods for the study of branching or debranching enzymes cannot therefore give quantitative information on changes in the proportion and distribution of 1,6-linkages in the substrate. Nevertheless, qualitative information can be conveniently obtained. In addition, determination of the

FIG. 4. *Effect of ammonium sulphate on the iodine complex of* Trichomonas gallinae *glycogen. Curves* 1 *and* 2 *were measured for half-saturated and quarter-saturated ammonium sulphate solution. Curve* 3 *shows the spectra in water.*

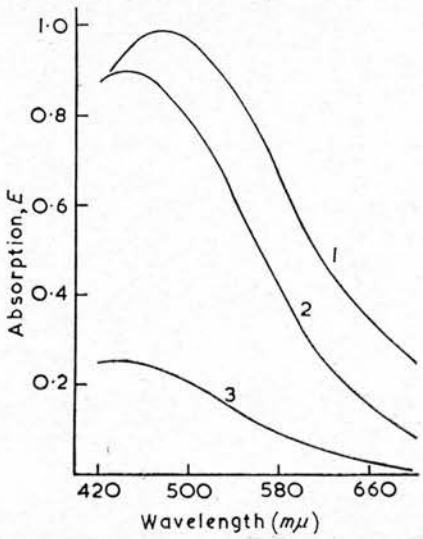
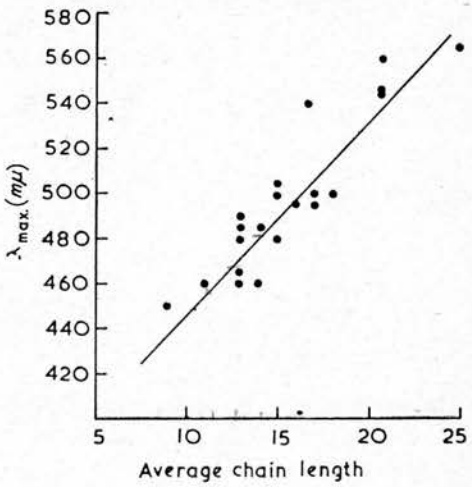


FIG. 5. *Relation between the average chain length of an amylopectin-glycogen type polysaccharide and* $\lambda_{max.}$ *of the iodine complex in half-saturated ammonium sulphate solution.*



$\lambda_{max.}$ of a polysaccharide–iodine complex, together with examination of other properties, may enable a distinction to be made between a "glycogen"- and an "amylopectin"-type polysaccharide. The results obtained so far are in good agreement with those from potentiometric iodine titrations.[15]

Effect of Ammonium Sulphate on Polysaccharide–Iodine Colorations.—The exact colour of a polysaccharide–iodine solution depends upon many factors,[20] but at constant temperature and with a constant concentration of reactants it may be increased by the presence of various salts, especially ammonium sulphate.[21]

Schlamowitz examined the absorption spectra of several glycogen–iodine complexes in 50% saturated ammonium sulphate under conditions in which a large excess of glycogen was present.[22] For many glycogens, $\lambda_{max.}$ was *ca.* 496 mμ, and was independent of $\overline{CL}$, although $E_{max.}$ appeared to be roughly proportional to the $\overline{CL}$ value. The significance of these results is lessened by the fact that $\overline{CL}$ values were obtained [22] by a periodate oxidation method [23] which was originally applied to amylopectins. With glycogens, this method does not give satisfactory results (cf. Manners and Archibald [8]). Using

Schlamowitz's conditions we measured $\lambda_{max.}$ and $E_{max.}$ for various glycogens, but were unable to find any relation between these properties and the $\overline{CL}$ (see Table 3).

In contrast to these results, addition of ammonium sulphate to 25% or 50% saturation in glycogen–iodine solutions, prepared as in Table 1, caused a marked increase in iodine-staining (Fig. 4, Table 4); *e.g.*, $E_{max.}$ values increased from 0·1 to 0·4 to the range 0·6—1·1. However, the solutions in 50% saturated ammonium sulphate became turbid, and a glycogen–iodine complex was slowly precipitated. (In one experiment, $E_{max.}$ fell from 0·87 to 0·82 within 15 minutes.) $E_{max.}$ values had, therefore, to be measured immediately after mixing. In later experiments, the stability of the solutions was increased by halving the glycogen concentration; this also halved $E_{max.}$ but did not affect $\lambda_{max.}$. In water or 25% ammonium sulphate solution, the glycogen–iodine solutions were clear and stable.

Glycogen β-dextrins were also examined; there was no appreciable change in $\lambda_{max.}$, although $E_{max.}$ increased (Table 4).

With amylopectin–iodine solutions, 50% ammonium sulphate caused only a slight increase in coloration; this was accounted for by an increase in $E_{max.}$ (from 0·9—1·2 to 1·1—1·6) rather than a change in $\lambda_{max.}$. Amylopectin β-dextrin behaved similarly.

Inspection of the results in Table 4 suggests that, with amylopectin and glycogen, $\lambda_{max.}$ is approximately related to the degree of branching; a correlation diagram is shown in Fig. 5. Since the length of the exterior chains is dependent on $\overline{CL}$, it follows that $\lambda_{max.}$ is also related to the exterior chain length. It may be possible to deduce $\overline{CL}$ values from measurements of $\lambda_{max.}$ under these conditions. By the method of least squares, with $\lambda_{max.}$ as the independent variable, these properties are related by the equation: $\overline{CL} = 16 + 0{\cdot}114\ (\lambda_{max.} - 500)$. The standard error in $\overline{CL}$ would be *ca.* 1·6 glucose residues. (We are indebted to Mr. A. G. Cock, Poultry Research Centre, Edinburgh, for this statistical analysis.)

It has been suggested [22] that ammonium sulphate facilitates iodine-complex formation by dehydration, providing a more hydrophobic environment for the iodine molecules. It is possible that a few of the longer B-chains in a glycogen molecule can, under these conditions, bind a limited amount of iodine by the helical mechanism rather than by adsorption.

Other salts, *e.g.*, magnesium sulphate, sodium sulphate, and sodium nitrate also increase the intensity of a glycogen–iodine coloration, but the effect is less than with ammonium sulphate.

Experimental

Most of the polysaccharide samples have been described elsewhere. We are grateful to Dr. G. O. Aspinall for the malt amylopectin, Dr. C. T. Greenwood for potato amylopectin IV, and Dr. J. R. Turvey for the *Zea mays* polysaccharides. The polysaccharide concentrations are based on the glucose content determined after acid-hydrolysis.

Effect of Polysaccharide Concentration on $\lambda_{max.}$.—Solutions containing severally 2·5, 1·5, and 1·0 mg. of horse muscle glycogen, 2·5 ml. of a 0·2% solution of iodine in 2·0% aqueous potassium iodide, and 1 drop of 3N-hydrochloric acid in a total volume of 25 ml. were prepared. In all three solutions, $\lambda_{max.}$ was 480 ± 5 mμ; $E_{max.}$ values were 0·25, 0·15, and 0·11 respectively.

Effect of Amylose as Impurity on Amylopectin–Iodine Solution.—Solutions containing potato amylopectin and 0, 5, or 10% w/w of potato amylose were prepared. The respective $\lambda_{max.}$ values were 555, 560, and 570 mμ, and $E_{max.}$ values 0·26, 0·28, and 0·33. The absorption of the amylose–iodine complexes alone was also measured. The effect was found to be additive.

Effect of β-Amylolysis on the Iodine-staining Power.—Digests containing rabbit liver VII glycogen and potato amylopectin (*ca.* 50 mg.) were incubated at pH 4·6 and 35° with barley β-amylase (2500 units) in a total volume of 50 ml. Samples (10 ml.) were removed at intervals, and heated to inactivate the enzyme. The conversion into maltose was determined, and equal *weights* of polysaccharide (≡ 2·3 mg.) stained with iodine and water or (≡ 1·15 mg.) stained with iodine and ammonium sulphate solution. Results are tabulated. If equal *volumes* of

polysaccharide–β-amylase mixture are stained with iodine, $E_{max.}$ decreases as the percentage conversion into maltose increases.

Time of incubation (hr.)	β-Amylolysis limit (%)	Water		Aq. $(NH_4)_2SO_4$	
		$\lambda_{max.}$ (mμ)	$E_{max.}$	$\lambda_{max.}$ (mμ)	$E_{max.}$
		Potato amylopectin			
0	0	540	1·1	540	0·7
0·5	61	540	1·5	540	0·6
2·0	64	540	1·6	545	0·6
29	65	545	—	540	—
		Rabbit liver glycogen			
0	0	450	0·3	490	0·5
0·5	41	425	0·2	440	0·3
2·0	51	410	0·2	425	0·4
29	55	430	0·1	425	0·4

Effect of Various Salts on the Iodine-staining Power.—Rabbit muscle III or *Mytilus edulis* VI glycogen (final concentration 0·005%) was stained with iodine in the presence of half-saturated solutions of various salts, with the tabulated results.

	Rabbit muscle glycogen		*Mytilus edulis* glycogen			Rabbit muscle glycogen		*Mytilus edulis* glycogen	
Conditions	$\lambda_{max.}$ (mμ)	$E_{max.}$	$\lambda_{max.}$ (mμ)	$E_{max.}$	Conditions	$\lambda_{max.}$ (mμ)	$E_{max.}$	$\lambda_{max.}$ (mμ)	E_{max}
Water	495	0·30	445	0·12	K nitrate	470	0·34	—	—
NH_4 sulphate	490	0·96	455	0·73	K sulphate ...	485	0·31	445	0·25
NH_4 nitrate ...	505	0·22	445	0·13	Na nitrate ...	—	—	450	0·35
Ca chloride ...	—	—	430	0·27	Na sulphate ...	485	0·35	445	0·29
Mg sulphate ...	495	0·50	440	0·51					

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Department of Chemistry, University of Edinburgh. [*Received, August 26th,* 1960.]

[1] Part X, Lawrie, Manners, and Wright, *Biochem. J.*, 1959, **73**, 485.
[2] Larner in "Methods in Enzymology," Academic Press Inc., New York, 1955, Vol. I, p. 222.
[3] Hobson, Whelan, and Peat, *J.*, 1951, 1451.
[4] Baldwin, Bear, and Rundle, *J. Amer. Chem. Soc.*, 1944, **66**, 111.
[5] Swanson, *J. Biol. Chem.*, 1948, **172**, 825; Bailey, Whelan, and Peat, *J.*, 1950, 3692; Kerr, Cleveland, and Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 3916.
[6] Archibald, Manners, and Wright, *Biochem. J.*, 1960, **75**, 10P; Liddle, Ph.D. Thesis, Edinburgh 1956; Archibald, Ph.D. Thesis, Edinburgh 1958.
[7] Fleming, Hirst, and Manners, *J.*, 1956, 2831.
[8] Manners and Khin Maung, *J.*, 1955, 867; Manners and Archibald, *J.*, 1957, 2205; Liddle and Manners, *J.*, 1957, 3432.
[9] Peat, Whelan, Hobson, and Thomas, *J.*, 1954, 4440.
[10] Chapman, Fells, and Chaikoff, *Experientia*, 1955, **11**, 283.
[11] Bell and Kosterlitz, *Biochem. J.*, 1935, **29**, 2027; Bell, *ibid.*, 1935, **30**, 2144.
[12] Van der Vies, *Biochem. J.*, 1954, **57**, 410.
[13] Peat, Turvey, and Evans, *J.*, 1959, 3341.
[14] Peat, Whelan, and Turvey, *J.*, 1956, 2317.
[15] Anderson and Greenwood, *J.*, 1955, 3016.
[16] Higginbotham, *Shirley Inst. Mem.*, 1949, **23**, 171.
[17] Thoma and French, *J. Amer. Chem. Soc.*, 1960, **82**, 4144.
[18] Peat, Whelan, and Thomas, *J.*, 1956, 3025; Liddle and Manners, *J.*, 1957, 4708.
[19] Cf. Greenwood and Rossotti, *J. Polymer Sci.*, 1958, **27**, 481.
[20] Morris, *J. Biol. Chem.*, 1946, **166**, 199.
[21] Sumner and Somers, *Arch. Biochem.*, 1944, **4**, 7.
[22] Schlamowitz, *J. Biol. Chem.*, 1951, **190**, 519.
[23] Potter and Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488.

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